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Malaria is a global health problem and remains a significant health problem for US military forces deployed to the tropics and subtropics. Existing antimalarial agents are either toxic or of diminished efficacy. We have discovered a new and novel class of antimalarial drugs which inhibit the process of heme polymerization. Extensive synthesis of analogs of the parent compound, X5, provided critical information on the structure-activity relationships which govern activity. Based on the knowledge gained from these studies we have now employed computer assisted drug design to produce drugs with enhance selectivity and potency.

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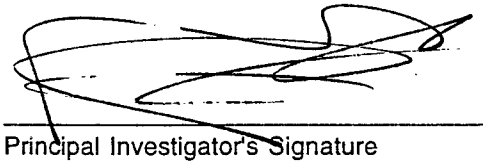

Principal Investigator's Signature

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Final Report-DOD SBIR contract # DAMD 17-97-C-7013

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1. Introduction

Malaria has plagued mankind through antiquity and remains the most significant parasitic disease in the tropics where it causes at least 200 million clinical episodes and claims 1 to 2 millions victims each year. As well as its impact on the civilian population of the world, malaria has had a major influence on military campaigns for thousands of years (72). Current measures for treatment of and prophylaxis against malaria still rely heavily upon chloroquine, quinine and the combination of pyrimethamine and sulfadoxine. While these classical drugs remain the standard of antimalarial chemotherapy, their usefulness is threatened by the spread of multidrug-resistant strains of *P. falciparum* and chloroquine-resistant *P. vivax*. Three antimalarials have been developed to counter drug-resistant *P. falciparum* infections including mefloquine, halofantrine, and artemisinin. Unfortunately, resistance has emerged to mefloquine and halofantrine and use of both drugs is complicated by a low therapeutic index due to neuropsychiatric and cardiotoxic side effects, respectively (51, 58)-effects which may limit their use in battlefield situations. Artemisinin and several related endoperoxides are among the most potent antimalarials ever developed but their usefulness is clouded by the discovery of neurotoxicity and a fatal neurologic syndrome produced by the drug in animals (10, 11, 86). Although these studies employed high concentrations of the drug and no such toxicity has been demonstrated in humans, reliance on artemisinin alone is associated with a relatively high rate of recrudescence (~10%) (57). And based on the known mode of action of the endoperoxides (i.e., their complexation with heme and subsequent formation of carbon centered radicals), we speculate that oral administration of the drug to humans could place the patient at a higher risk of developing esophageal and stomach cancer. Taken together, with a worldwide resurgence in the incidence of malaria, the spread of multidrug-resistant strains of *Plasmodium falciparum*, the emergence of chloroquine resistant *P. vivax*, and the increasing resistance of *Anopheline* mosquitoes to insecticides, malaria continues to be an enormous threat to US military personnel deployed to the tropics and subtropics (38).

It is true that the great panacea for malaria therapy is development of a long-lasting vaccine. However, until this becomes a reality (noting the recent disappointing failure of the SPf66 malaria vaccine (60)) we must rely on a dwindling arsenal of antimalarial drugs. Development of a safe, effective, novel antimalarial agent with a unique mechanism of action capable of treating multidrug

resistant forms of *P. falciparum* and *P. vivax* remains the overall goal of our work. The rationale behind our approach is the selective targeting of the parasites' Achilles' heel, the acidic vacuole (61), through administration of a diphenyl-bridged prodrug which undergoes "cyclo-activation" within this compartment to become a potent antimalarial tricyclic agent.

Herein we present a specific example of the general principle of our drug design strategy. Based on our findings, we propose exifone and pentahydroxyxanthone (X5) as lead compounds for development as antimalarial agents-the former (and its analogs) as prodrugs which offer an alternative method of drug delivery to enhance a drug's pharmacologic properties or to reduce unwanted side effects of the desired tricyclic compound. The purpose of this proposed Phase I project was to synthesize compounds (designed according to our rationale) in sufficient quantity and purity to allow WRAIR experts to confirm our *in vitro* findings, to gain insight into the mode of action of the xanthone-based drugs, and to evaluate the potential of our approach to chemotherapy in appropriate models of malaria and other protozoan parasites of military importance, e.g., *Leishmania* ssp. In order to fully appreciate the significance of findings made by Interlab scientists during this collaborative SBIR Phase I work supported by the DOD, it is first necessary to present background information relating to the biology and biochemistry of malarial parasites.

2a. Body of Proposal

Background Information

***Plasmodium* life cycle.**

Malaria is caused by protozoans (*Plasmodium*) that specifically parasitize erythrocytes. The disease begins with the bite of a mosquito and injection of *Plasmodium* sporozoites, which then invade liver parenchymal cells, transform into exoerythrocytic parasites, and undergo rapid multiplication (schizogeny). Exoerythrocytic schizonts release merozoites, which invade erythrocytes and initiate repeated cycles of development. Some of these differentiate into sexual forms which upon ingestion by a mosquito, undergo further development into sporozoites thus completing the malarial life cycle (for review see (85)).

The *Plasmodium* digestive vacuole.

The digestive vacuole is an acidic proteolytic compartment central to the metabolism of the plasmodia and generally regarded as the parasites' Achilles' heel (Schematic in Figure 1). In this vacuole hemoglobin is degraded to provide amino acids for parasite growth (61). While most of the heme-iron produced during the hemoglobinolytic process is polymerized into insoluble hemozoin ("malarial pigment"), some iron is released for incorporation into essential parasite ferroproteins. The most potent antimalarials are believed to act here. Quinoline drugs such as chloroquine and quinine accumulate in the acidic vacuole (31) and are believed to inhibit polymerization of heme and formation of hemozoin (23, 24, 82). However, this action has recently come into question (56).

Heme polymerase.

In 1992, Slater and Cerami (75) presented evidence that the polymerization of free heme into hemozoin was an enzyme-mediated process. They reported detection of a novel heme

polymerase and demonstrated that the enzyme was inhibited by chloroquine. Subsequently, others have demonstrated that the polymerization of heme could be carried out in a chemically facile manner in a test tube in the total absence of parasite proteins (23, 24). This led to speculation that formation of malarial pigment was a spontaneous, not an enzyme mediated process. Even chemical polymerization of heme was inhibited by chloroquine. More recently, Pandey and Tekwani (64) demonstrated that "hemozoin" formed in vitro in the standard acetate buffer system was not chemically identical to hemozoin but probably an insoluble acetate salt of heme. Thus, all that can be said at present is that chloroquine can inhibit the formation of this heme:acetate salt. Herein we present evidence that polymerization of heme occurs in dilute phosphate buffer yielding a polymer which appears to be chemically identical to hemozoin (in Experimental Section). Under the physiologically relevant conditions of our assay (i.e., 20mM phosphate buffer, pH 5.2, 37°C, 25μM heme), hemozoin formation was potently inhibited by xanthones, which are the focus of our research, but NOT by either chloroquine or quinine. Thus, as it has been for over half a century, the mechanism of action of the quinoline-based antimalarials remains unclear. One possible mode of action for the quinolines that is often over-looked is inhibition of vacuolar ATPase activity (17).

Oxidant stress and malaria parasites.

Oxidant stress describes a situation where chemical or metabolic generation of oxygen-derived radicals exceeds normal defense mechanisms (33). During the processes of hemoglobin degradation and hemozoin formation, heme iron is oxidized from ferrous to ferric state. This process results in generation of oxygen radicals and is believed to cause considerable oxidative stress on the parasite. To mount a defense against these radicals, the parasite sequesters superoxide dismutase from the host cell and transports it into the digestive vacuole (25, 26). This enzyme converts superoxide to hydrogen peroxide which is then cleaved by a catalase activity. It is apparent that while this scavenging process and other related antioxidant defense mechanisms serve to protect the parasite, the balance must be a tenuous one, as parasitized red blood cells (PRBC) are known to be very susceptible to oxidant stress (33). Not surprisingly, ideal conditions for *in vitro* cultivation of the parasite are microaerophilic e.g., 1.5-3% O_2 (74) but the parasite must survive in the human host where the capillary and venous level of oxygen tension is in the range of 11-15% (84).

Oxidant drugs.

A group of therapeutic agents collectively referred to as oxidant drugs hold the promise for effective treatment of multi-drug resistant *Plasmodium* parasites (81). These drugs cause enhanced production of oxygen radicals inside parasitized erythrocytes (33) or act to render parasites (or their host cells) more susceptible to oxygen radical attack. Accordingly, antimalarial oxidant drugs are structurally diverse and include seemingly unrelated compounds such as redox-cycling agents (i.e., hydroxy-naphthoquinones (47) and active metabolites of primaquine (6, 43)), antagonists of glutathione metabolism (i.e., buthionine sulfoximine (80)), methylene blue (3, 4), ascorbic acid (53, 54), and artemisinin (57, 81) (Figure 2).

Primaquine, an oxidant drug.

Special attention has been given to primaquine (an 8-aminoquinoline) which is extremely important to malaria chemotherapy because of its activity against several life-cycle stages of the *Plasmodium* parasite (55). It is the only agent used which is active against the primary tissue schizonts, thus functioning as a causal prophylactic agent; against exoerythrocytic forms, thus curing relapsing forms of malaria; and against the gametocytes, thus blocking transfer to the mosquito vector. And when it is present in blood (i.e., one of its metabolites), it also inhibits the development of sporozoites in already infected mosquitoes. Indeed, while it is often overlooked, primaquine also exhibits a significant action against blood stages of the parasite (71). However, there are practical problems associated with administration of primaquine which relate primarily to its toxicity and the necessity for prolonged use in radical treatment schedules. Most significantly, this drug is known to induce hemolytic lesions in glucose 6-phosphate dehydrogenase-deficient patients, a relatively common genetic condition among inhabitants of malarious regions of the world.

The mode of action of primaquine is not well understood; however it is believed that both hemolytic activity and antimalarial action are linked to redox-active metabolites of the drug which generate oxidant stress on the parasite and host cells (6, 13, 43, 50) (Figure 2). Meshnick and coworkers (39) have recently suggested that primaquine would be more useful if its oxidant effects on erythrocytes could somehow be diminished. They point out that one way to accomplish this is the simultaneous administration of an antioxidant, an oxygen radical scavenger. As is evident from our findings described below and in recent manuscripts (87, 89), we predict that exifone will act synergistically with primaquine *in vivo* to enhance its antimalarial activity (exifone + oxygen radicals will lead to xanthone formation in parasitized cells) and to reduce its hemolytic action on uninfected red cells (exifone is a highly efficient oxygen radical scavenger). Isobolar analysis to study the interaction between exifone and the redox active primaquine metabolite, 6-hydroxy-5-methoxy-8-aminoquinoloine, vs. *P. falciparum* is stifled by the lack of a reliable synthetic route for the drug.

2b. Experimental Design and Findings

In the balance of this report we describe the discovery of a compound which, we believe, functions as a prodrug and exhibits a profound synergistic antimalarial response when combined with an oxidant agent. This represents the discovery of a novel mechanism for drug delivery in which a prodrug is induced to undergo pre-programmed cyclization to become an active antimalarial agent within the parasite. If true, one could engineer a seemingly limitless array of prodrugs to deliver structurally and mechanistically distinct compounds to counter emerging drug resistance phenotypes. In our Phase I proposal we stated that the products of the prototypic transformation described below, xanthone analogs, represent a novel class of antimalarial agents which act in distinct fashion to kill malarial parasites. We presented preliminary results indicating that xanthones act by inhibiting the process of heme polymerization. Here, we present more convincing evidence that this is the primary mode of action of the xanthones and that heme is indeed the primary target within the parasite. This information allows us to predict specific design modifications to the tricyclic compounds to enhance their selectivity and potency as

antimalarial agents. Such information together with results from experiments leading to our discovery of xanthones as selective antimalarial agents are described in detail in this section of the report.

Rufigallol as an antimalarial agent.

We screened a series of hydroxyanthraquinones for antimalarial activity. The assays were conducted with our 72 hr assay measuring the incorporation of ^{3}H-ethanolamine into parasite lipids to monitor parasite growth. Rufigallol was the most potent of the 12 anthraquinones tested yielding an IC_{50} of 226±31nM as judged from the average of 14 experiments (88).

Exifone as an antimalarial agent.

Exifone was synthesized because of its structural resemblance to rufigallol. Because it lacks one of the keto moieties of the corresponding anthraquinone (and thus the internal aromatic ring capable of redox cycling) we predicted that the compound would exhibit inferior activity relative to rufigallol. Indeed, the compound exhibited only weak antiplasmodial activity (IC_{50} ~ 4.1μM) (89). The prior clinical use of exifone in humans for the treatment of Alzheimer's disease and Parkinson's disease as well as reports indicating that the drug may produce liver toxicity in a small fraction of the test population have been summarized (89).

Synergism between rufigallol and exifone.

Standard isobolar analysis was employed for evaluating synergism between the various drug combinations of rufigallol and exifone. In combination, potent synergism was observed (Figure 3) (89). Calculating the degree of potentiation by geometric means of an isobole yielded a value of ~60-fold. The rufigallol/exifone drug pair demonstrated even greater potency against cultures of synchronized parasites at the trophozoite stage of development. Under these conditions (i.e., initiating the experiment with trophozoites) a combination of 1nM rufigallol with 10nM exifone produced an IC_{50} response. Figure 3 shows the accentuated concave curve of the isobole yielding a geometric value for degree of potentiation of ~300-fold. We have performed the same experiment with two other strains of *P. falciparum*, W2 (a multidrug resistant clone) and OLU1 (a recent isolate originating from Ibadan, Nigeria/Dr. Oduola's group) and the data are virtually identical with the data presented for the D6 strain above (87, 89). Other benzophenones were tested by similar fashion for potentiation of rufigallol (vs. the D6 strain). As compared to the profound effect of combining exifone with rufigallol, most of these compounds did not significantly impact the anti-plasmodial activity of rufigallol. From our structure vs. activity analysis, it is apparent that proper positioning of free hydroxy groups around the benzophenone nucleus and the presence of an ortho hydroxy group are important structural features for the drug synergy.

Hypothesis: Exifone is transformed into a tricyclic xanthone by the pro-oxidant activity of rufigallol.

We hypothesized that increased susceptibility of *P. falciparum* to rufigallol in the presence of exifone could be explained as follows: rufigallol enters PRBC leading to the formation of hydrogen peroxide in fashion similar to the well-documented redox cycling behavior of hydroxy-naphthoquinones and hydroxyanthraquinones (22, 83). In the presence of the vast quantities of

"adventitious" iron (or iron chelates) (2) the hydrogen peroxide produced is readily decomposed to hydroxyl radicals as formulated by Haber and Weiss (5, 32). These highly reactive radicals attack exifone and transform the diphenyl compound into a potent tricyclic antiparasitic agent, 2,3,4,5,6-pentahydroxyxanthone (X5) (Figure 4) (89).

Synergism between exifone and ascorbic acid.

Exifone and ascorbic acid act synergistically to inhibit the growth of *P. falciparum* in vitro (Figure 5) (87). While this result may seem surprising since ascorbic acid is considered an antioxidant in humans, Marva et al. (54) have demonstrated its prooxidant activity in PRBC. It is believed that this activity results from an intra-erythrocytic Haber-Weiss reaction occurring in the acidic food vacuole of the parasite wherein iron and heme are liberated as hemoglobin is digested (28). Accordingly, ascorbate enters the infected cell and serves to reduce iron ($Fe^{+3} \Rightarrow Fe^{+2}$) and oxygen (formally: $O_2 \Rightarrow \bullet O_2^- \Rightarrow H_2O_2$). The resulting ferrous iron induces the conversion of hydrogen peroxide to yield highly reactive hydroxyl radicals which cause destruction of macromolecules (36). We speculated that hydroxyl radicals generated in this fashion also led to enhanced formation of the putative antimalarial xanthone from exifone (Figure 6). However, there is no consensus among researchers in the field as to whether hydroxyl radicals formed under such conditions [i.e., the "Udenfriend system" (79)] are responsible for aromatic hydroxylation or if this phenomenon occurs through the intermediacy of a reactive ferryl-oxygen complex (5, 52). Regardless of the actual mechanism involved, it is noteworthy that the typical plasma concentration for vitamin C in healthy individuals is between 28-85μM with stable levels approaching 100μM for individuals taking 0.5 - 3 gm of supplemental vitamin C per day (44).

Effects of oxygen tension on exifone's antimalarial activity.

The synergistic interaction between ascorbic acid and exifone is consistent with Udenfriend's system of ascorbate-accelerated Fenton reactions. Note that this well-characterized chemical reaction also requires oxygen. We therefore speculated that higher oxygen tension would potentiate exifone's antimalarial activity as such conditions accelerate formation of hydroxyl radicals from ascorbic acid (through its interaction with heme iron in the acidic vacuole) and enhance conversion of exifone to X5. Results from 4 independent experiments each conducted in duplicate have confirmed our prediction. The potency of exifone was only marginally improved when oxygen tension was increased from 1.5% (IC_{50} ~1 μM) to 15% (candle jar) (IC_{50} ~0.75 μM) (Figure 7) (87). However, oxygen tension had a dramatic impact on the potency of exifone when incubated with ascorbic acid (tested here at a physiological level of 100μM) where the IC_{50} value for exifone decreased to 0.02 μM (5 ng/ml) (virtually identical to chloroquine's *in vitro* activity).

Exifone: *in vivo* toxicity studies-historical aspects.

In the late 1970's and throughout the early 1980's, studies focused on the use of exifone in treatment of cognitive decline associated with age in geriatric and Parkinsonian patients (1, 20, 46, 68). Experiments conducted in patients and in animals indicated that exifone improved memory function without producing a spontaneous effect on motor activity (1, 66, 67). As part of these experiments it was noted that an oral dose of 1024mg/kg exifone did not produce measureable

toxicity in geriatric patients. In 1989, it was discovered that continued administration of high doses of the drug (600 to 1,000mg, administered one to three times daily for 2 to 6 months) to elderly patients caused liver damage in some patients which reversed with discontinuation of exifone (16, 30, 34, 48, 49, 63, 65). The incidence of detectable liver damage was reported to be in the range of 1/15,000 patients (48). In at least one case, exifone was believed to cause such liver toxicity that the patient died and the drug was apparently removed from human trials. It is still not clear from any of these cases that exifone produced liver damage directly or in combination with other medications which the patient had been taking (e.g., many of the patients were also taking ginkgo extract and other herbal remedies). Longterm dosing with exifone alone in animal models did not produce detectable liver damage. Regardless, while the longterm use of high doses of exifone may produce reversible liver toxicity in humans, we believe that short term administration of even higher doses of exifone would be safe for treatment of acute cases of malaria. And even lower doses may be effective in combination with an oxidant drug to produce safe prophylaxis from disease. However, because of the safety concerns mentioned above our longterm goal is to chemically modify exifone to improve its antimalarial properties and remove any potential for liver toxicity.

Conversion of exifone to a pentahydroxyxanthone *in vitro*.

Exifone was incubated with ascorbic acid in the presence of iron in a buffered solution stirred vigorously in a wide-mouthed beaker, open to the air (to introduce oxygen into the system) at 39-42°C (12, 52, 79). The concentration of reactants was as follows: 0.1 M sodium acetate (pH 5.0), 0.8 mM EDTA, 1.0 mM exifone, 3.5 mM ascorbic acid and 0.8 mM $FeCl_2 \cdot 4H_2O$) in a total volume of 20 ml. At the beginning of the experiment and after 45 minutes of incubation 2 ml samples of the reaction mixture were withdrawn and transferred to acid-washed glass vials, frozen and lyophylized. The residue was taken up in acetone (2 ml) and heated at reflux overnight in the presence of excess potassium carbonate (0.5 gm) and dimethylsulfate (1 ml). At this point, 0.2 ml of each sample was mixed with 0.8 ml of acetone and the protected reaction products were subjected to gas chromatography-mass spectrometry (1 µl sample injected, 25 meter DB5 column, temperature gradient program: 50°C/4 min, 18°C/min, 280°C/18.22 min, Hewlett-Packard Model 5970MS/HP 5890GC). The total ion chromatogram and mass spectra of the reaction products were collected and are presented in Figure 8 (87). Assignment of the chemical identity for each peak shown in the figure was made based on a comparison to the retention time and mass fragmentation pattern of chemically synthesized material. As shown, after 45 minutes of incubation we observed the conversion of exifone (i.e., detected as the hexamethoxy protected form, Peak #1) to the putative X5 intermediate, 2,3,4,5,2',3',4',-heptahydroxy-benzophenone (E7, Peak #2) and another peak appearing at 25.8 minutes of the chromatogram and corresponding to authentic X5 (Peak #4). These reaction products were not detectable in control conditions in which either ascorbic acid or iron was excluded. Note: Peak #3 is a mixture of the other two isomers of E7.

Antimalarial activity of X5 and structurally related xanthones.

X5 was synthesized and subjected to antimalarial testing (87). For comparison, we also purchased several commercially available xanthones and synthesized additional xanthones and

subjected them to *in vitro* antimalarial testing. Xanthones were dissolved in DMSO at a concentration of 10mM and diluted in complete medium to provide 10X stock concentrations of the drug in the range of 1nM to 100μM. Results showed that X5 acted similarly toward the mefloquine-resistant D6 strain and the multidrug resistant W2 strain with IC_{50}'s in the range of 0.4μM (Table 1). Fully protected penta-methoxy-X5 exhibited an IC_{50} value more than 2 orders of magnitude greater than X5, thereby indicating a role for the free hydroxy groups in the antimalarial effect. Since it was possible that X5's antimalarial activity was affected by the highly acidic nature of the 3 and 6 position hydroxyls (40, 41), we converted X5 to penta-acetyl X5 with acetic anhydride. Whereas the methylether protecting groups are known to be very stable, the acetyl groups are more labile towards chemical or enzymatic hydrolysis, and it follows that penta-acetyl X5 could yield free X5 within the parasite. As shown in Table 1, penta-acetyl X5 was more potent than X5 yielding an IC_{50} value of ~20nM vs. D6 and W2. The enhanced activity of penta-acetyl X5 relative to X5 and penta-methoxy X5 suggests that the unprotected compound is chemically unstable above neutral pH. Subsequent tests have demonstrated that there is no synergistic interaction between X5 (or acetyl-X5) and rufigallol or ascorbic acid, data consistent with our "xanthone hypothesis" (87).

In search of the minimal structural requirements for the antimalarial activity amongst the hydroxyxanthone derivatives, we synthesized and tested selected hydroxyxanthones. Xanthone, 3-hydroxy-xanthone, and 3,6-dihydroxyxanthone did not exhibit antimalarial activity, whereas 4,5-dihydroxyxanthone demonstrated activity of ~20μM (roughly equivalent to the in vitro activity of minocycline and doxycycline in our system). In summary, the data indicate that the simple 4,5-dihydroxy analog possesses the minimal structural features of the xanthone necessary for the antimalarial effect.

Mode of action of X5: Complex formation between heme and X5.

Based on structural features of X5, we predicted that it would form a complex with free heme. We used UV/visible difference spectroscopy to measure the optical signal produced upon interaction between heme and X5. The actual methods employed were described in our first monthly report and a publication relating to this issue will appear in July of this year (42). Dual tandem cuvettes allowed direct comparison of the same amounts of heme and X5 mixed in the sample cuvette and separated in the reference cuvette. By this experimental design the contributions from slight differences in the heme, X5 and dimethylformamide concentrations to the difference spectra were cancelled, i.e., only the effects of complexation are observed. Figure 9 shows the family of UV/visible difference spectra induced by binding of X5 to heme over 45 minutes of incubation. The spectra contain a difference peak at 270 nm which decreased with time, a dip at 327 nm, and shoulders at ≈250 and ≈420 nm which increased with time. These changes are indicative of the red shifts in the UV (240-260 nm) and visible (320-400 nm) absorbance produced upon formation of the heme-X5 complex. Interestingly, in preliminary experiments in which the samples were kept at 37°C (note: the experiment described above was conducted at 4°C to retard polymerization), we detected by visual inspection the formation of a flocculent brown precipitate in the heme control sample within 1 hour of incubation, while no such phenomenon was observed in the test sample containing both heme and X5 (Figure 10).

The precipitate was washed twice with deionized water and once with methanol, and characterized by means of differential solubility, elemental analysis and infrared spectroscopy. For the purpose of comparison we also prepared the heme-precipitate in low and high ionic strength acetate buffer (the latter corresponding to the method of preparation of so-called "β-hematin"). As shown in Table 2, the phosphate-derived material exhibited properties characteristic of hemozoin ("malarial pigment"). It was found to be insoluble in methanol, ethanol, dimethylsulfoxide, 2.5% sodium dodecylsulfate, and a solvent mixture of methanol/acetic acid/water (8:1.5:0.5), and immobile on thin layer chromatograms developed with this mixture (64, 76). The polymer also exhibited an additional infrared absorbance band ($\approx 1650 cm^{-1}$) indicative of the presence of a carboxylate coordination to iron (76). Elemental analyses showed that the percentages of carbon, hydrogen, nitrogen and iron in the 0.02 M phosphate and 0.02 M acetate derived products corresponded closely to the values reported for hemozoin (27). The 4 M acetate derived product had an elemental composition consistent with that of a hematin-triacetate adduct (42). In all, these data suggest that the precipitate formed upon incubation of hemin in phosphate buffer is a heme polymer chemically analogous to hemozoin and distinct from the product formed upon incubation of hemin in acetate buffers (24, 64).

Heme polymerization and its inhibition by X5.

Since the preliminary results indicated that X5 inhibited heme polymerization, we developed an assay based on the spectrophotometric detection of soluble heme. Briefly, a 10 mM stock solution of hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 1 hour. X5 and related xanthones were dissolved in dimethylformamide at 10 mM and diluted into 10 ml of pre-warmed phosphate buffer to a final concentration of 25 μM. Polymerization was initiated by addition of 25 μl of the hemin stock solution to the test sample to yield a final concentration of 25 μM heme. 25 μl of dimethylformamide was added to the control sample. After 7, 30, 60, 120 and 210 minutes of incubation at 37°C, a 1 ml aliquot was withdrawn, transferred into an Eppendorf tube, and centrifuged at 14000g for 2 minutes at room temperature to pellet the polymer. The soluble fraction was then transferred to a semi-microcuvette (polymethylacrylate, VWR), and the absorption was measured at 360 nm against a blank of the test compound in buffer. Control experiments indicated that the amount of dimethylformamide used in this assay did not significally affect the rate of polymerization.

To estimate the effect of test compounds (X5) on heme polymerization at a given time of incubation, the percentage of soluble hemin was calculated using the following formula:

$$\% \text{ soluble hemin} = \frac{A_{(\text{drug + hemin) t}} - A_{(\text{drug) t}}}{A_{(\text{hemin) t = 0}}} \times 100\%,$$

where $A_{(\text{hemin) t=0}}$ was determined by addition of 5 µl of the hemin stock solution to 2 ml of pre-warmed phosphate buffer placed directly into a cuvette and followed immediately by measuring the absorption at 360 nm against a blank of buffer.

The dose-dependent inhibition of heme polymerization was evaluated as follows: dilutions of a given drug were made into test tubes containing 2 ml of pre-warmed phosphate buffer (0.02 M, pH 5.2). Then 5 µl of the hemin stock was added to each tube to yield a final concentration of 25 µM. The reactions were allowed to proceed for 2 hours in a 37°C waterbath. After incubation, the polymer was pelleted as described above and the absorption (360 nm) of each soluble fraction was measured against a blank containing the drug alone in buffer.

To obtain the 50% inhibitory concentrations (IC_{50} values), the percent inhibition of heme polymerization was calculated using the following formula:

$$\% \text{ inhibition of polymerization} = \frac{(A_{(\text{drug+hemin)}} - A_{(\text{drug)}})_{\text{2hrs.}} - A_{(\text{hemin) 2hrs.}}}{A_{(\text{hemin) t=o}} - A_{(\text{hemin) 2hrs.}}} \times 100\% .$$

The IC_{50} values were determined graphically by plotting the percent inhibition of heme polymerization vs. drug concentration.

Under the conditions of our assay, heme polymerization was pH-dependent (pH 4.5-5.5) (Figure 11), occurred spontaneously, and was more than 95% complete within 2 hours of incubation (Figure 12, Panel A) (42). Addition of one equivalent of X5 resulted in complete inhibition of polymerization (Figure 12, Panel B). Addition of X5 to polymerized heme did not reverse the process. This, as well as the ability of X5 to alter the spectral properties of the heme, strongly suggests that X5 inhibits heme polymerization through the formation of a soluble complexes with heme monomers and oligomers.

Inhibition of heme polymerization by known antimalarial agents.

We evaluated known antimalarials (e.g., chloroquine, primaquine, quinacrine, artemisinin, and methylene blue) as inhibitors of heme polymerization under our *in vitro* assay conditions. As shown in Table 3, we found that the addition of 1 to 40 equivalents (chloroquine was tested at 400 equivalents! 10mM) of these compounds had no effect on the rate of *in vitro* polymerization, as determined spectrophotometrically. It is noteworthy that past spectroscopic studies designed to monitor complex formation between heme and chloroquine were conducted at or above neutral pH-not at the acidic pH of the vacuole (3, 8, 9, 18, 59). We therefore decided to investigate the possibility that chloroquine co-precipitates with the heme polymer, as shown

recently by Sullivan et al. (27, 77) in intact parasitized red cells. We monitored the concentration of chloroquine by measuring its absorption at 340 nm in the presence of an equimolar concentration of polymerizing heme (25 μM). Indeed, we found that the concentration of soluble chloroquine decreased ~35% following 2 hours of incubation, indicative of the chloroquine/hemozoin co-precipitation phenomenon-the precipitate so formed was slightly green in character. Similar spectroscopic studies were then performed with other antimalarial agents. Primaquine, quinacrine and methylene blue, which are all positively charged under mildly acidic conditions also co-precipitated with the heme polymer (producing distinctive changes in the color and character of the polymer-especially in the last case), possibly due to association with free carboxyl groups of the heme polymer and π-π interactions between the aromatic systems. Clearly, heme polymerization is distorted but not inhibited in our in vitro assay or in vivo in parasite infected red cells (56) treated with chloroquine (i.e., pigment clumping"). It must be stated that addition of chloroquine to polymerizing heme under our conditions leads to the formation of a greenish-brown precipitate-possibly indicating the presence of μ-oxo dimer formation (14) as suggested by Dr. Jonathon Vennerstrom at the recent meeting of the ASTMH.

Inhibition of heme polymerization by hydroxylated xanthones.

Structure-activity relationships were determined for xanthones as inhibitors of spontaneous heme polymerization and as antimalarial agents (Table 1). The IC_{50} values are the average of at least two independent determinations of full dose-response curves. Experiments were conducted on trophozoite synchronized parasites following two cycles of sorbitol lysis (and an incubation of 21hrs after the last sorbitol treatment). Xanthone and the tested monohydroxyxanthones did not exhibit any inhibitory activity in our assay. Moderate inhibitory activity (i.e., $IC_{50} \approx$ 8-20 μM) was observed for the compounds bearing a single hydroxy group at either 4- or 5-position, whereas the greatest activity was observed for xanthones containing hydroxy groups at both positions. For example, 2,3,4-trihydroxyxanthone exhibited an IC_{50} of 16.5 μM, while 2,3,4,5,6-pentahydroxyxanthone (X5) yielded a value of 1.2 μM. Consistent with this structure-activity profile, the 4,5-hydroxylated xanthones also exhibited the most pronounced *in vitro* antimalarial activity (Table 1). On closer inspection of the data, one will notice that isomers bearing hydroxy substituents at the peri-position (with respect to the carbonyl of the xanthone) were less active than a corresponding xanthone without this substitution pattern (i.e., even if either 4 or 5-position was hydroxylated). For example, 1,3,5-trihydroxyxanthone, was found to be completely ineffective in our heme polymerization assay ($IC_{50} > 100\mu M$) and without activity against *P. falciparum* in vitro at 60μM, the highest concentration tested (vs. 16.5 μM for the 2,3,4-trihydroxy derivative). This result, combined with similar isomeric comparisons drawn from the Table of data (i.e., 1,3,5,6,7-pentahydroxyxanthone vs. X5), indicates that the presence of a hydroxy group at either 1 or 8 position of the xanthone (the so-called *"peri"* or *"ipso"* positions) deactivates the compound-perhaps through intramolecular hydrogen bonding with the carbonyl oxygen. This interaction likely diminishes the electron density around the carbonyl oxygen and decreases the likelihood for coordination to heme iron.

Taken together, our SAR analyses indicates that X5 forms soluble complexes with heme monomers or oligomers and interferes with hemozoin formation. Such action may prevent

detoxification of free heme, starve the parasite for iron, or significantly increase the osmotic pressure within the parasite digestive vacuole. In this regard it is to be noted that the polymerization process must sequester all or most of the freed heme, which otherwise would accumulate to a concentration of up to 0.4 M (77). The relative abilities of X5 and some of its analogs to inhibit *in vitro* heme polymerization are in good correlation with their *in vitro* antimalarial activities, and are indicative of the following structure-activity relationships: (i) in general, a higher degree of hydroxylation is favored for the inhibitory activity (presumably to enhance the solubility of the complex); (ii) hydroxylation at 4- and 5-positions appears to be absolutely critical, and (iii) hydroxylation at the peri-position has a negative influence on both activities. Based on these observations, we developed a model for a possible docking orientation of a symmetrical polyhydroxyxanthone (X6) to heme (Figure 13) displaying several significant interactions: (1) between the heme iron and the carbonyl oxygen; (2) between the two planar aromatic systems; and (3) between the carboxylate side groups of the heme and the 4- and 5-position hydroxyls of the xanthone. Importantly, this model predicts that chemical modifications at the 4- and/or 5-positions which improve association with the heme carboxylate groups will result in even greater antimalarial activity. Hydroxylation at either peri-position of the xanthone probably diminishes the electron density at the carbonyl oxygen (due to intramolecular hydrogen bonding) of the xanthone, thereby decreasing its affinity for heme iron.

Additional SAR analysis of 4,5-substituted xanthones and xanthone congeners.

Isosteric replacement of the ring oxygen with a sulfur to produce 4,5-dihydroxythioxanthone was effected by Dr. Winter through a novel route analogous to his unique xanthone synthesis procedure. The compound was prepared because, unlike the corresponding xanthone which is planar, computer modeling indicates that the thioxanthone does not have a planar configuration. Based on the computer modeling we anticipated (correctly) that the compound would be less effective in the heme polymerization assay with inferior antimalarial activity relative to that of 4,5-dihydroxyxanthone. It appears from this preliminary result that there would be little advantage in further pursuit of 4,5-disubstituted thioxanthones as antimalarial agents which act through inhibition of heme polymerization. We are in the process of synthesizing the corresponding acridone to allow a side-by-side examination of which tricyclic nucleus exhibits the greatest inhibitory activity in both systems (i.e., in the context of the 4,5-dihydroxy conformation).

Drug design.

Based on our model of drug action, replacement of or extension from the hydroxyl moieties of 4,5-dihydroxyxanthone with alkylhydroxy, alkylamines, carboxylic acids or amidines may significantly improve the antimalarial activity of the tricyclic agent. Accordingly, such modifications should yield a xanthone analog with enhanced heme binding properties, greater solubility within the acidic vacuole, and targeted accumulation within the vacuole. We therefore prepared 4,5-bis-(β-diethylaminoethoxy)xanthone (45-DEAE-X) (Figure 14). This compound represented the most direct and least expensive route to a xanthone with the desired characteristics (more sophisticated computer modeling will be needed to optimize geometries). The substituted xanthone is a diprotic base like chloroquine. On entry into the acidic vacuole, the side chains become positively charged, effectively "trapping" the drug within this

compartment where it complexes with heme. The positively charged residues are designed to be in opposition to the heme carboxylate side chains so as to facilitate formation of a soluble heme:xanthone complex (geometries and energy minimizations limited to that possible with SCULPT for Macintosh). We predicted that the ionic nature of the trapped xanthone would maintain the drug:heme complex in solution. An early preparation of 45-DEAE-X yielded only 6mgs of material which was consumed during confirmatory chemical analyses. A larger scale preparation was recently effected by Dr. Winter which yielded ~120mgs of white glassy flakes of pure material. It is currently being tested in vitro as an inhibitor of heme polymerization and as an antimalarial agent and the data will be transmitted to WRAIR scientists as soon as it is generated. We shall compare its activity to that of the simple 4,5-dihydroxy analog.

Structurally related compounds as inhibitors of heme polymerization.

We screened several structurally related hydroxy-coumarins (including 4-hydroxycoumarin), flavones (including quercetin and 7,8,3',4'-tetrahydroxyflavone), and chalcones (including 2',3',4'-trihydroxychalcone) for inhibition of heme polymerization. None of these compounds exhibited activity at concentrations as high as 100μM. However the widely distributed natural product ellagic acid (Figure 15) was a potent inhibitor-possessing roughly one quarter of the activity of X5. The IC_{50} for ellagic acid vs. *P. falciparum* (D6) in our standard assay is ~0.1 to 0.2μM. Owing to the widespread distribution of this compound in the plant world (21, 37, 70), it is of importance to ascertain if its presence in plant extracts (i.e., from cinchona bark or the Chinese wormwood) contributes to the combined antimalarial efficacy of traditional decoctions.

Anti-leishmanial activity of synthetic and naturally occuring xanthones.

The pharmacologic significance of xanthones as secondary plant metabolites has not been fully explored (78) but many of the recent reports focus on their activity against monoamine oxidase activity (7, 41). However, investigators have found that xanthones exhibit activity against a variety of bacteria (e.g., *Staphylococcus aureus* and *Mycobacterium tuberculosis*), fungi (*Candida albicans*), and cultured cancer cell lines *in vitro*. Based on the antimalarial action exerted by our synthetic xanthones against *Plasmodium* parasites and the knowledge that xanthones are present in plant extracts used in traditional remedies used throughout the tropics, we tested xanthones against promastigotes of *L. donovani* and *L. mexicana* by the established ^{3}H-thymidine incorporation method of WRAIR investigators (35). The strains were obtained from Drs. Buddy Ullman and Scott Landfear (OHSU). The compounds demonstrated IC_{50} values in the low micromolar range in the 72hr assay (Table 4). Mangostin was the most potent xanthone in our preliminary screening with *in vitro* activity roughly 1000-fold greater than the standard therapeutic agent for leishmaniasis, stibogluconate (the latter compound being synthesized in this lab by Dr. Winter). The simple xanthone, X5, was slightly less active than mangostin. Here it is noteworthy that leishmania parasites are unable to synthesize heme or the tetrapyrole porphyrin ring system and must therefore obtain these essential cofactors from the host macrophage (15, 29, 62). As the parasites reside as amastigotes within the acidic phagolysosome of the macrophage, the drug design scheme planned for improving the antimalarial potency of xanthones may also yield compounds with selective antileishmanial activity-due primarily to starvation of the parasite for porphyrins and heme.

Xanthones form complexes with porphyrins.

We have now conducted preliminary studies on the ability of various xanthones to complex with the porphyrins, coprotoporphyrin I and coprotoporphyrin III. The interaction between the drug and porphyrin was studied by employing the method of uv/visible scanning spectroscopy. Complex formation was indicated by spectral shifts when either of the porphyrins was incubated with an equimolar concentration of X5 or 4,5-dihydroxyxanthone (red shifts in the Soret region of 5-10nm). However, the most significant spectral shifts occurred on addition of 4,5-bis-(β-diethylaminoethoxy)xanthone to the tested porphyrins. Shown in Figure 16 (panel A) are the spectra taken from an experiment in which 12.5μM coprotoporphyrin I was incubated with 4,5-DEAE-X for 2 minutes (20mM phosphate buffer, pH 7.2, 37°C)-note the 25nm bathochromic shift in the Soret band. An impressive shift is also observed when the drug is added together with coprotoporphyrin III under the same conditions (Panel B). Analogous experiments conducted under mildly acidic conditions (pH 5.0) also demonstrate complex formation. Taken together, our analyses indicate that xanthones can bind to porphyrins as well. It is therefore apparent that these compounds (i.e., suitably substituted xanthones) possess the necessary biochemical activity to restrict parasite access to both heme and to porphyrins. In addition to their potential use as antiparasitic agents, it is also not to be overlooked that xanthones may prove useful in treatment of porphyrias-a family of disorders related to abnormal porphyrin metabolism and to multiple chemical toxicity (19, 73). In these clinical conditions it is possible that xanthones may be effective in mobilizing porphyrins, which have accumulated in blood and tissues, and enhance their excretion. At the very least (based on our spectroscopic evidence), these compounds should lessen the degree of photosensitivity exhibited by patients stricken with cutaneous symptoms of porphyria (due in part to the decreased energy of the light absorbed by the drug:porphyrin complex).

3. Conclusions and summary comments.

The importance to medicine of natural products lies not only in their chemotherapeutic effects but also in their role as lead molecules for production of new drugs with enhanced properties. Quinine from the cinchona bark, for example, which continues to be used for treatment of malaria, has also served as a template molecule for design of numerous drugs such as quinacrine and chloroquine. More recently, artemisinin from the plant *Artemisia annua*, has served in this capacity for design of more potent, chemically-accessible antimalarial trioxanes. These examples highlight a typical path to new drug discovery especially in the field of antimalarial drug development, that is, identification of a natural product with the desired activity followed by lead drug optimization.

Our course to discovery of xanthones as antiparasitic agents did not follow the normal route. We were drawn into an investigation of their activity by the discovery of a potent antimalarial synergism between exifone (a benzophenone) and oxidant drugs. From this interaction we anticipated that 2,3,4,5,6-pentahydroxyxanthone (X5) was formed inside parasitized cells and that this compound represented the true antimalarial priniciple responsible for the synergism. Along our path to discovery we have: 1) established the synergistic antimalarial activity of exifone in combination with two structurally distinct oxidant drugs, 2) demonstrated enhanced

antimalarial activity of exifone in combination with ascorbic acid under conditions of increasing high oxygen tension, consistent with the role of oxygen in the Udenfriend system, 3) demonstrated the chemical transformation of exifone into X5 *in vitro* (with the appearance of a putative intermediate, heptahydroxybenzophenone) under conditions known to exist in the digestive vacuole of the malarial parasite, 4) conducted extensive structure-activity testing of benzophenones which indicate that exifone acts synergistically while other hydroxy-benzophenones do not, 5) demonstrated that exifone is a highly effective hydroxyl radical scavenger, 6) synthesized X5 and demonstrated its strong antimalarial activity and 7) demonstrated that X5 does not act synergistically with either ascorbic acid or rufigallol. Taken together, our findings are consistent with the notion that formation of the xanthone follows free radical hydroxylation of exifone, loss of a molecule of water, and ring closure to form the tricyclic structure. As this reaction occurs readily *in vitro* under mildly acidic conditions at 37°C and only in the presence of iron, we are convinced that the exifone $\Rightarrow$ xanthone transformation occurs in the parasite food vacuole which contains free iron and has an internal pH between 4.7 and 5.4 (90). Our most recent studies, conducted as part of this Phase I project, combine to indicate that once it is formed, X5 binds to free heme and prevents heme polymerization. The net effect of this blockade is accumulation of a huge concentration of soluble heme:X5 complexes and a corresponding increase in the osmotic pressure within this compartment. The extent of this accumulation should lead to swelling, and ultimately, to the lysis of the acidic food vacuole. Vacuolar swelling and fragmentation are also hallmarks of chloroquine treatment of parasitized red blood cells (45), however, we believe that mechanism by which this is brought about is distinct from the xanthone-mediated drug action. This speculation is based merely on the fact that X5 is at least as active against chloroquine-resistant strains as sensitive strains.

Direct inhibition of hemozoin formation by a xanthone-based antimalarial compound may also facilitate our immune system in defending against malaria infection. When the intraerythrocytic schizonts rupture and release merozoites, hemozoin disperses in host vasculature, eventually being ingested by circulating monocytes and resident macrophages. Parasitized erythrocytes are also directly phagocytized by phagocytic cells. Accumulation of hemozoin-laden macrophages is commonly observed in the spleen and liver during malaria. The production of oxygen radicals (the so-called oxidative burst) and reactive nitrogen intermediates by macrophages seems to play a central role in defense mechanisms against malarial parasites (69). Hemozoin is known to impair production of these radicals by macrophages and accumulation of this material in these cells may significantly suppress the immune response to malaria. Thus it is conceivable that the xanthone-based antimalarials which we are designing to specifically inhibit heme polymerization may simultaneously kill parasites and restore immune function. Results from in vivo experiments will serve as an early indication of the clinical utility of these novel compounds.

We believe that our discovery of the synergistic combination of exifone with an oxidant drug has highlighted a novel mechanism of directed cell killing which relies on the specificity of the oxidant agent. It follows that other bridged two-ring systems may be affected in similar fashion and that one could design a prodrug which, in combination with an oxidant agent, would be transformed *in situ* into an even more potent tricyclic antimalarial compound-perhaps

minimizing any side effects which may be derived from direct administration of the tricyclic compound. Thus, while we describe this phenomenon for the transformation of a benzophenone to a xanthone, one could specifically design bridged molecules to undergo the cyclodehydrative process to become tricyclic phenothiazines, substituted xanthones, acridones, acridines, or thioxanthones and including variants of these which are designed to specifically inhibit hemozoin formation (i.e., xanthones, acridones, and thioxanthones) or vacuolar proton-ATPase activity, subvert oxidant defense mechanisms (i.e., methylene blue), modulate ion transport, intercalate into DNA (acridines) or interfere with nucleic acid processing.

One of our original proposals was to evaluate and compare the prodrug delivery of an antimalarial xanthone (i.e., by coadministration of exifone with a redox active primaquine metabolite) vs. direct delivery of the tricyclic compound remains untested due to difficulties in preparation of the metabolite. We remain committed to this study and fully intend to develop useful, reproducible methods for preparation of 5-hydroxy-6-methoxy-8-aminoquinoline as well as the corresponding 5-acetoxy-derivative. The concept of our novel approach to drug design and delivery will likely spur parallel advances in the chemotherapy of other human diseases currently treated with oxidant drugs and perhaps clinical conditions wherein there is a need to prevent the inappropriate deposition of heme.

So what? In our original Phase I proposal to the DOD prepared in the summer of 1996 we had just completed a study designed to investigate the complexation of heme by our lead xanthone, X5. From this successful series of experiments we also discovered the unexpected phenomenon of heme polymerization in vitro and the fact that X5 inhibited this process. This information was "stitched" onto the end of our proposal together with an overlay diagram of one possible orientation of the xanthone docking onto heme. To put our docking model to the test we proposed to synthesize a panel of hydroxyxanthones. We did so by a novel facile route which Dr. Winter of Interlab developed. Each xanthone was tested as an inhibitor of heme polymerization and this information was correlated to its antimalarial activity. Within the group of hydroxyxanthones there was an impressive correlation between the two activities. Many of the compounds were also been tested by WRAIR and the data confirmed and extended our own (i.e., the importance of 4 and 5 position hydroxyls and the deactivating effect of 1 and 8 position hydroxyls). WRAIR Expt'l Therapeutics also confirmed that suitably substituted xanthones (i.e., X4) were at least as effective against a multidrug resistant clone of *P. falciparum* (W2). Based on results from our Phase I studies we now profile 4,5-bis-substituted xanthones as the next generation of xanthone-based antimalarial agents. Based on our own chemical knowledge of xanthone synthesis, we anticipate that these compound will be simple and inexpensive to produce from 4,5-dihydroxyxanthone (which we have already prepared and evaluated). For example, a pound of 4,5-bis-(diethylaminoethoxy)xanthone should cost less than $100 to produce (an especially important aspect considering that malaria is primarily a disease of poor under-developed countries). Like other xanthones, this compound should be stable to extremes of pH and temperature (and time!...they are found in fossils) and therefore suitable for oral administration. It has not escaped our attention that the 4,5-bis-substituted xanthone, specially designed to form a soluble complex with heme, may also exert inhibitory effects on other pathogens or parasites which require access to or sequestration of heme or the porphyrin ring for

survival. We thank you for the invitation to apply for continued financial support for this collaborative work with DOD officials under the SBIR Phase II program. We remain firmly convinced that our work will lead to development of a long-lasting therapy for malaria. The financial support and collective intellectual support provided by the DOD during this 6-month Phase I project was essential to our success.

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5. Appendices

Personnel Statement: The following individuals have received support from contract DAMD 17-97-C-7013: Rolf W. Winter, PhD, Michael K. Riscoe, PhD, and David J. Hinrichs, PhD.

Figures and Tables

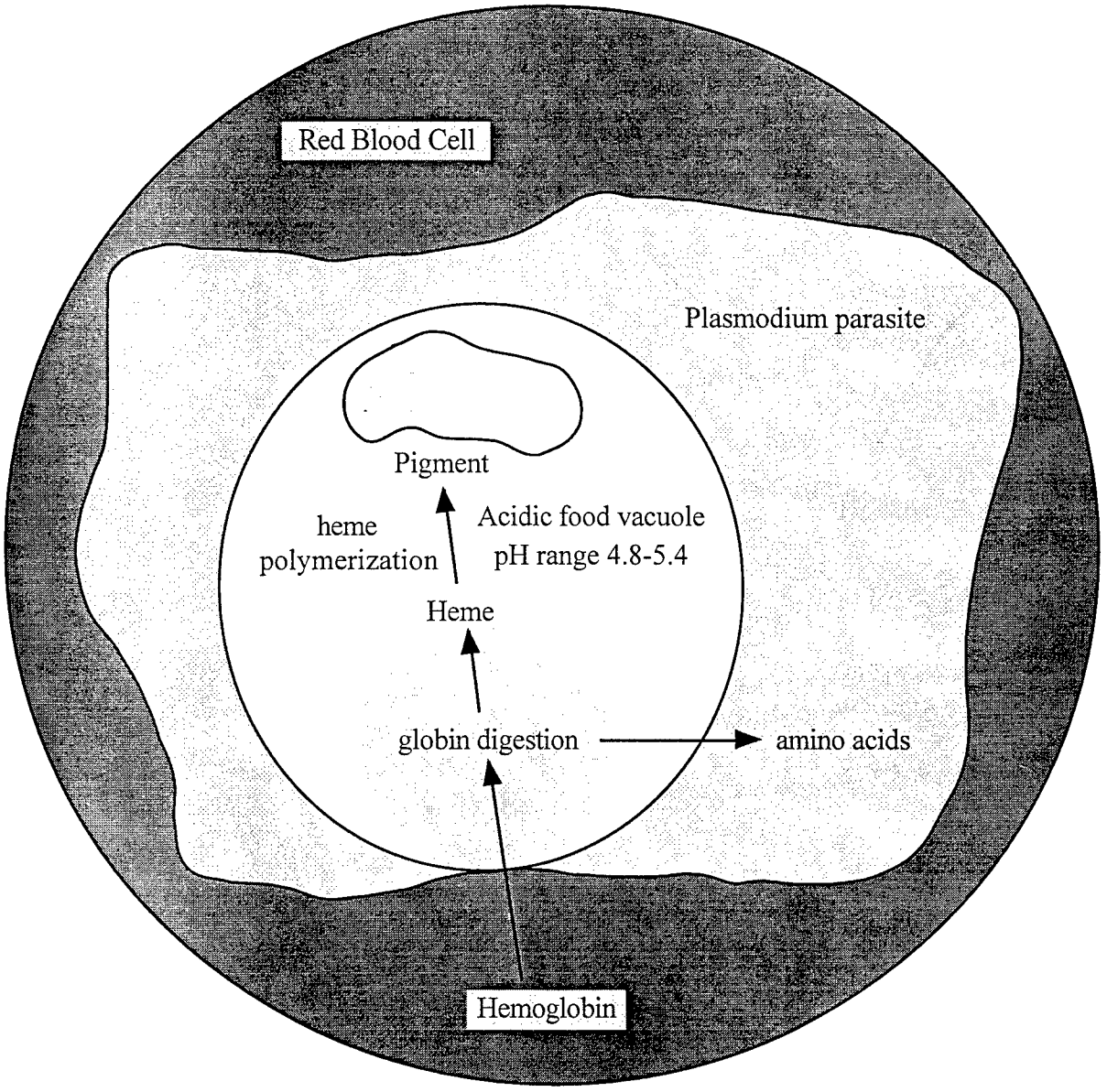


Figure 1. Schematic depiction of hemoglobin digestion (with the concommitant release of heme) by the intracellular parasite, *Plasmodium falciparum*.

Figure 2. Chemical structures of putative antimalarial oxidant drugs.

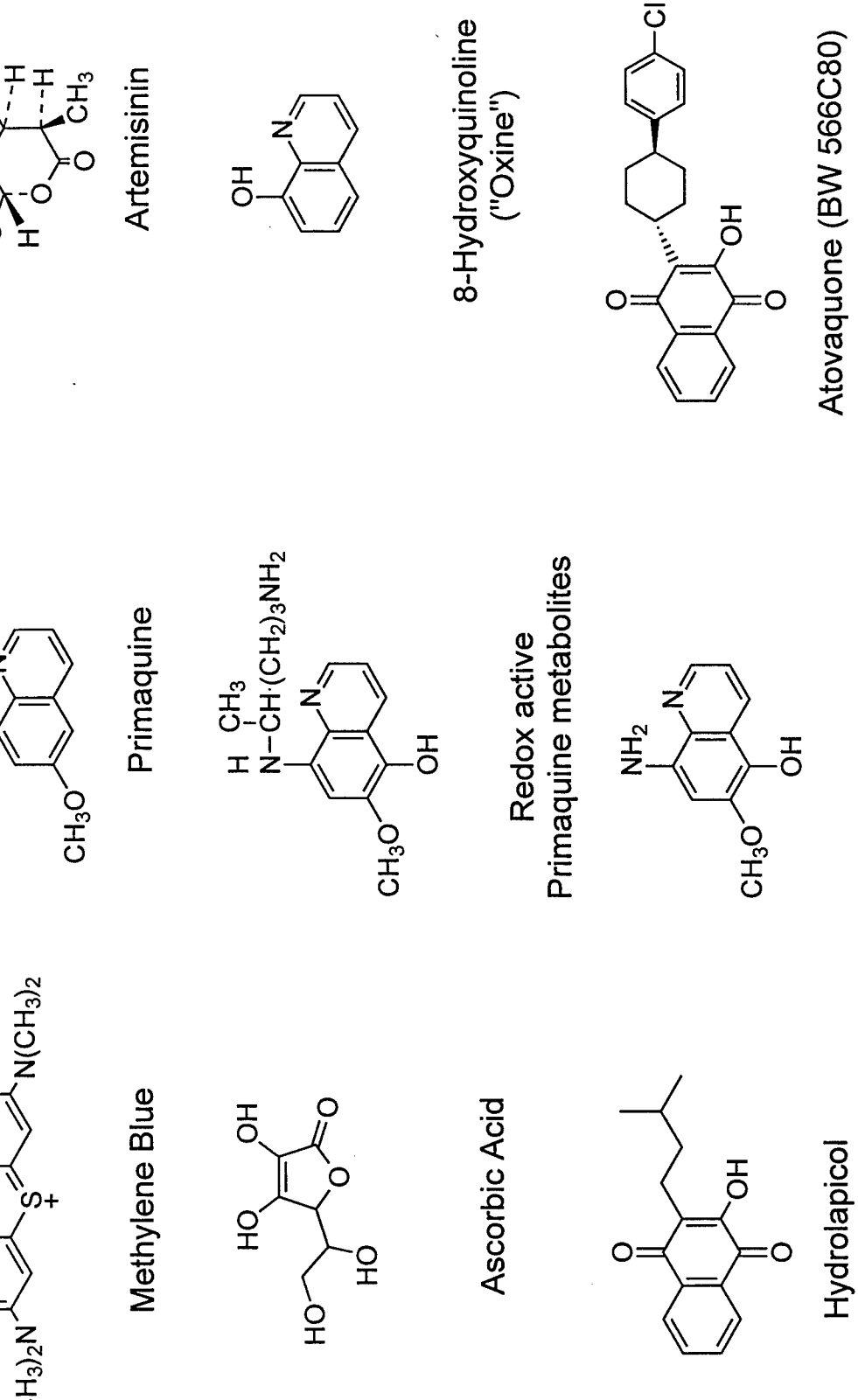


Figure 3. Isobolar analysis of the synergistic drug combination, rufigallol and exifone against the D6 clone.

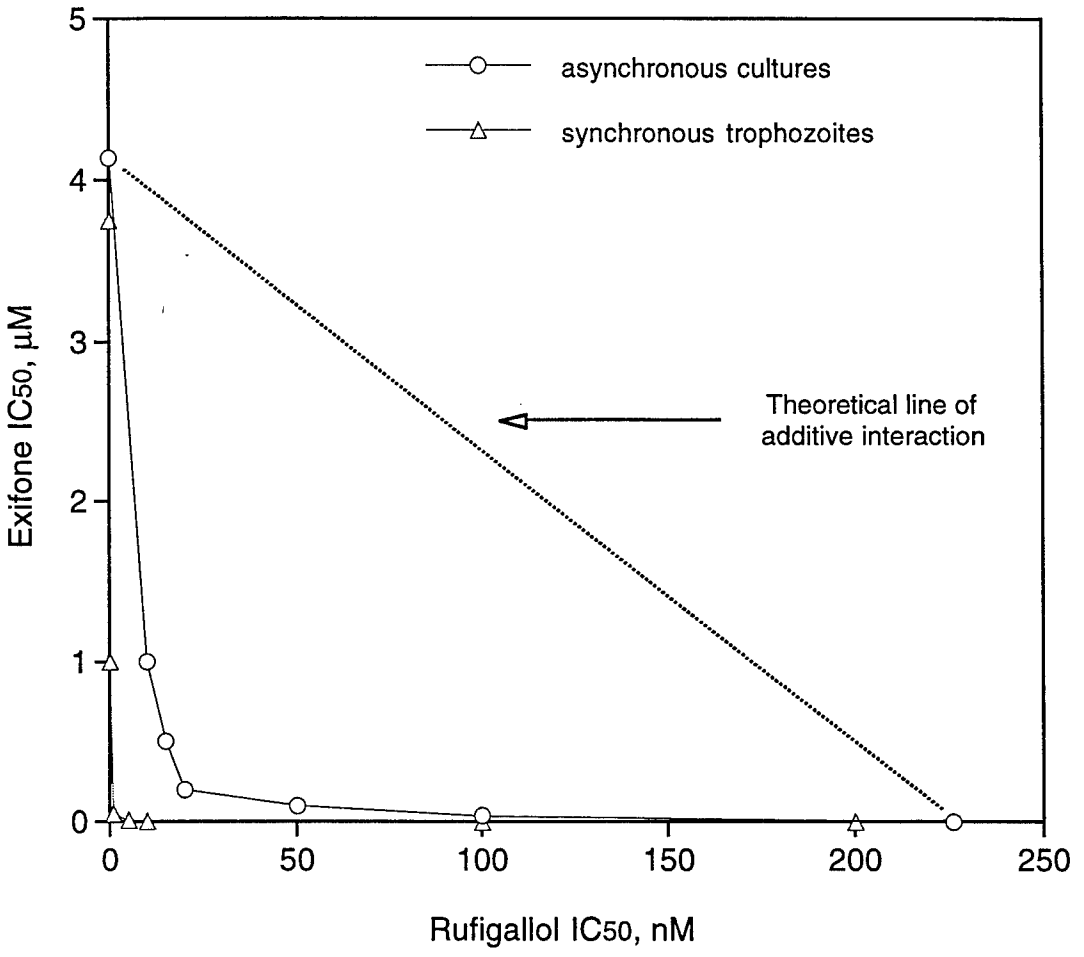
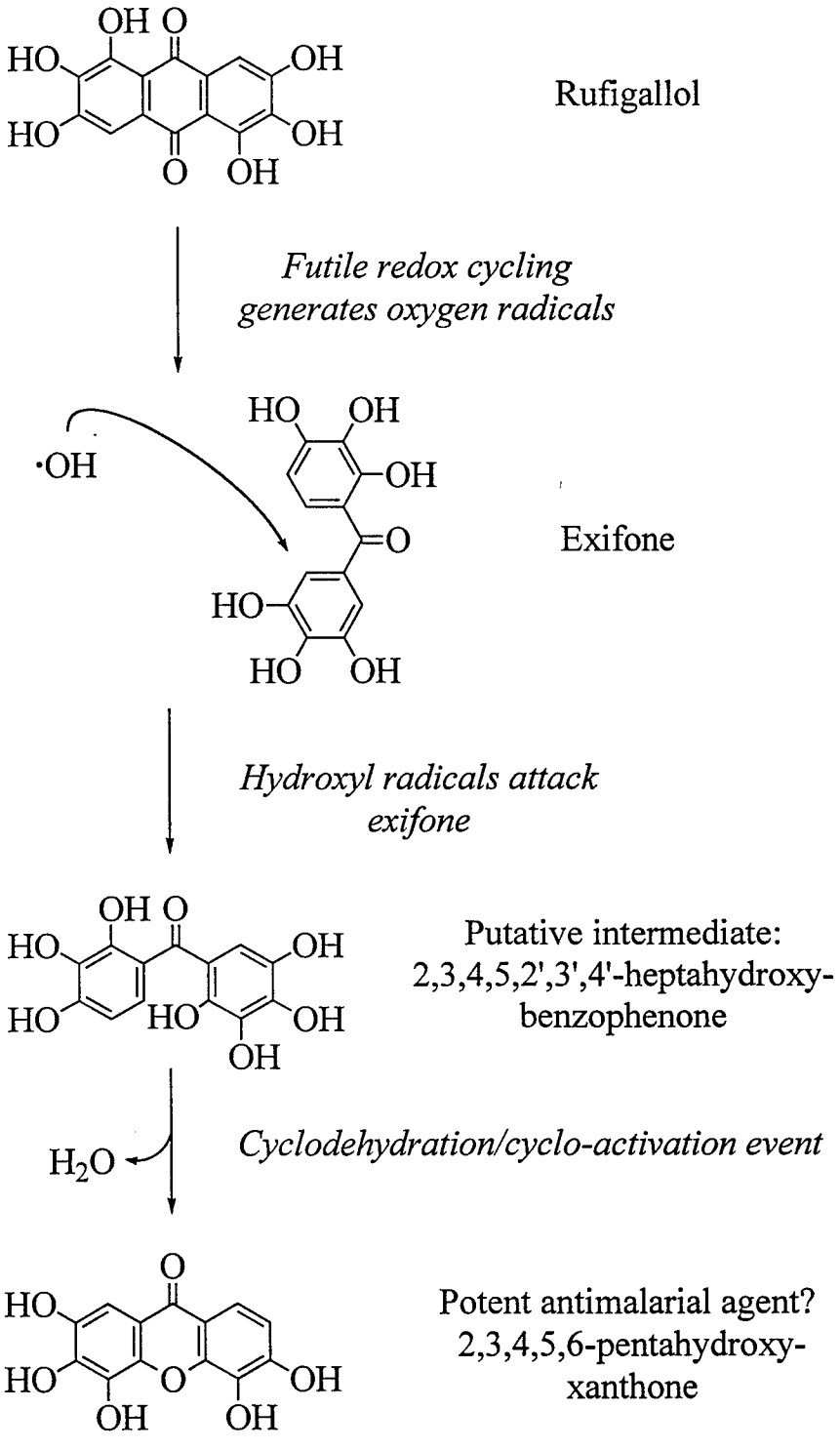


Figure 4. Possible mechanism for potentiation of rufigallol by exifone.



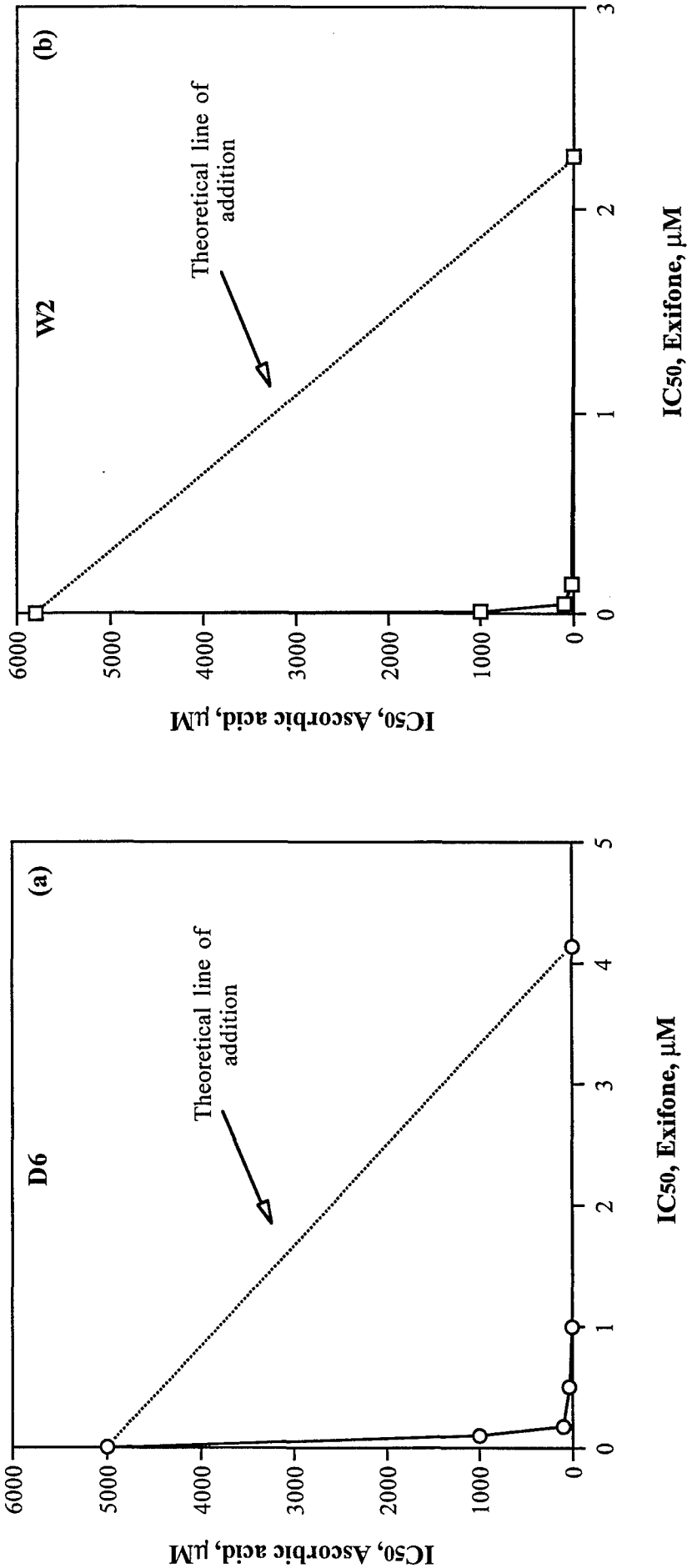
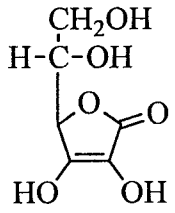


Figure 5. Isobolar analysis of exifone and ascorbic acid against the D6 clone of P. falciparum (A) and the multidrug resistant clone, W2 (B).

Figure 6. Proposed Mechanism Underlying the Synergism Between Ascorbate and Exifone

Step 1.



ascorbic acid

$$+\ Fe^{+++} +\ O_2 \longrightarrow \text{dehydroascorbic acid} +\ Fe^{+++} +\ [H_2O_2]$$

Step 2.

$$[H_2O_2] +\ Fe^{++}\ \text{(free heme in parasite vacuole)} \longrightarrow\ Fe^{+++} +\ OH^- +\ ^{\bullet}OH$$

Step 3.

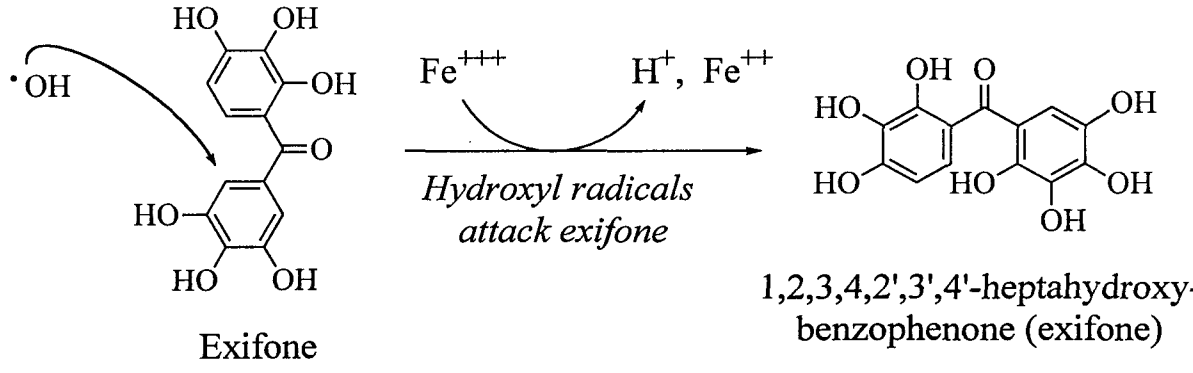
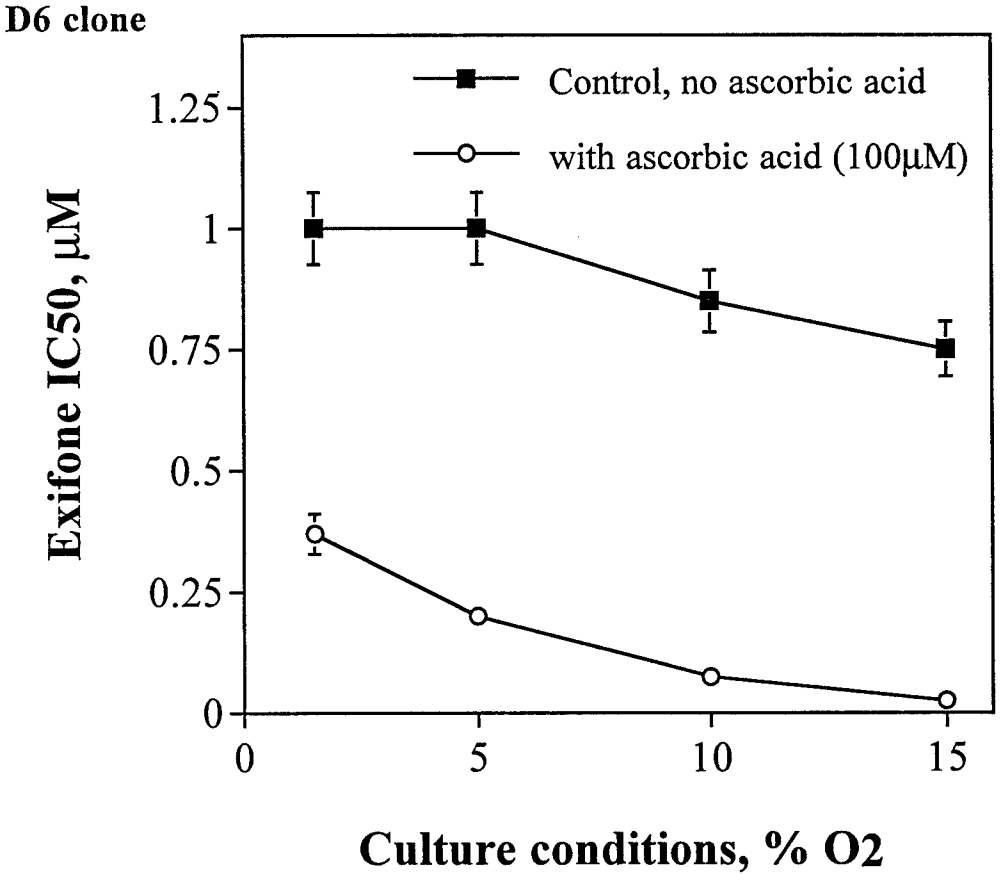


Figure 7. Effect of oxygen on the antimalarial synergism between exifone and ascorbic acid.



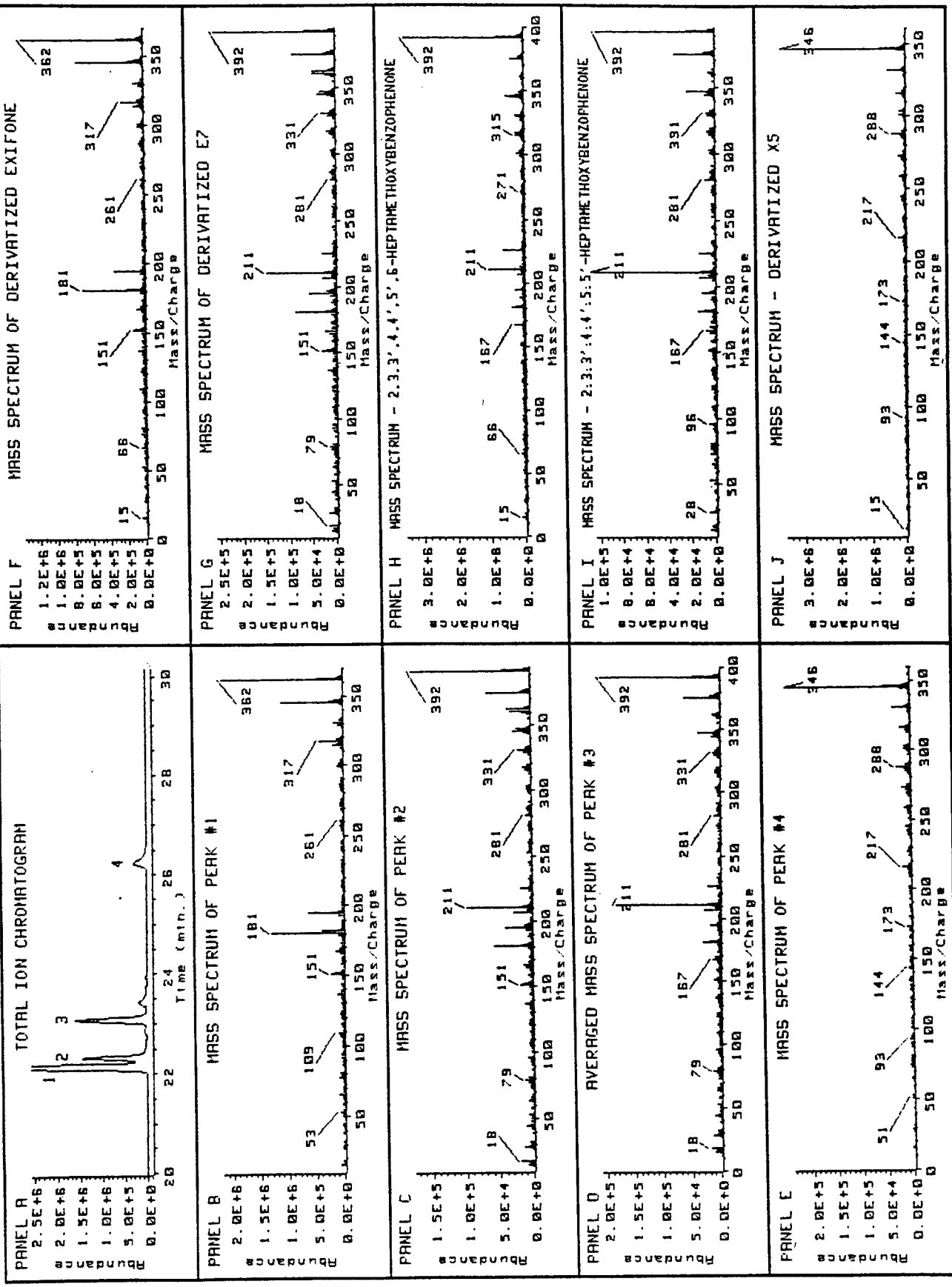


Figure 8. Panels A-J. Conversion of exifone into pentahydroxyxanthone (X5) under conditions of the Fenton reaction as detected by GC-MS (derivatized to their corresponding permethylether form prior to injection). Panels: A. total ion chromatogram of reaction products, B. mass spectrum of peak #1, C. mass spectrum of peak #2, D. mass spectrum of peak #3, E. mass spectrum of peak #4; Standards: F. mass spectrum of derivatized exifone, G. mass spectrum of derivatized E7, H. mass spectrum of 2,3,3',4,4',5',6-heptamethoxy-benzophenone, I. mass spectrum of 2,3,3',4,4',5,5'-heptamethoxybenzophenone, and J. mass spectrum of derivatized X5.

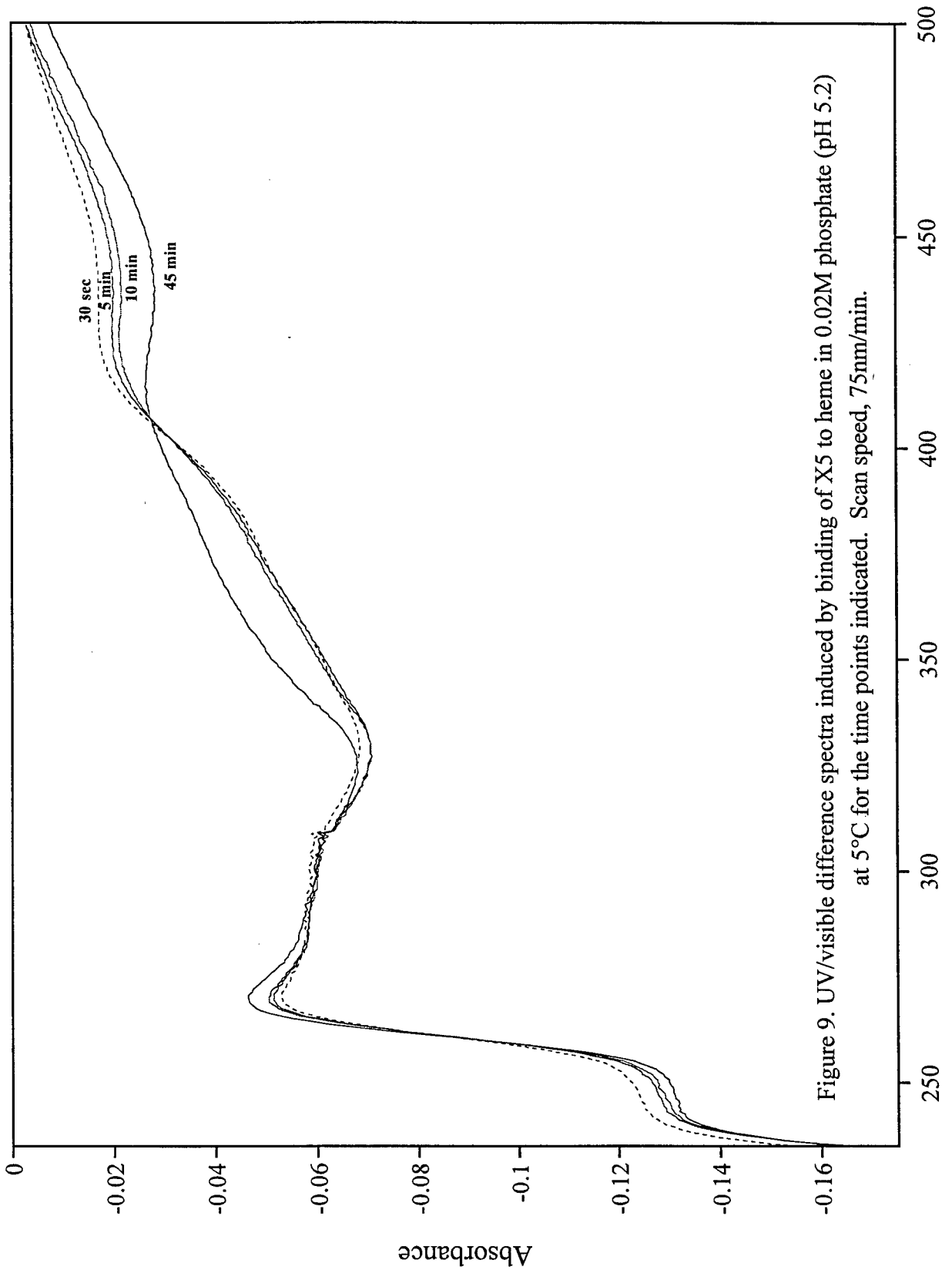


Figure 9. UV/visible difference spectra induced by binding of X5 to heme in 0.02M phosphate (pH 5.2) at 5°C for the time points indicated. Scan speed, 75nm/min.

Fig. 10 In vitro Heme Polymerization Assay

Conditions as described in text)

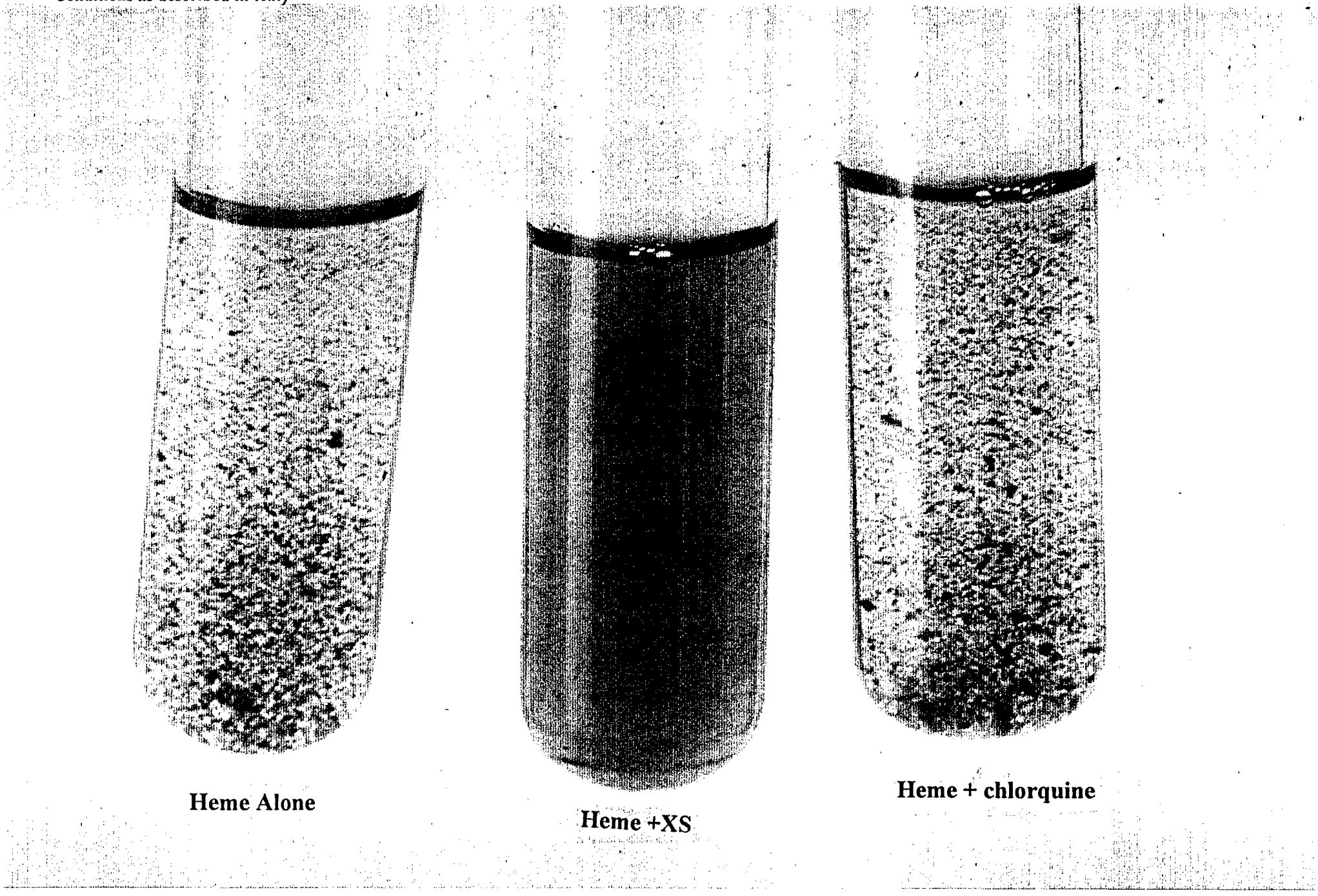
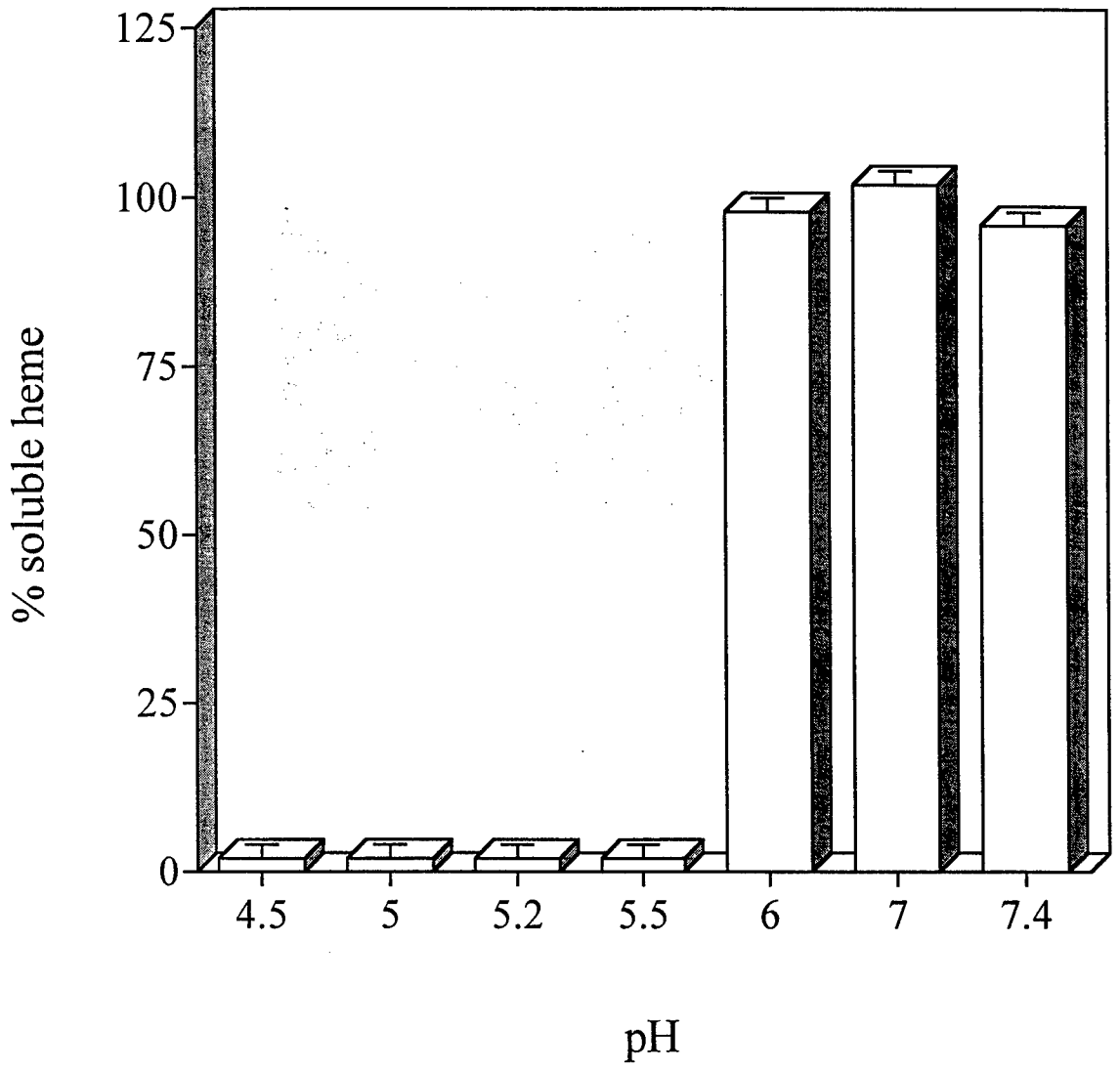


Figure 11. pH profile for in vitro heme polymerization in 0.02M phosphate (37C, 2hrs of incubation). Values are the mean of duplicate determinations.



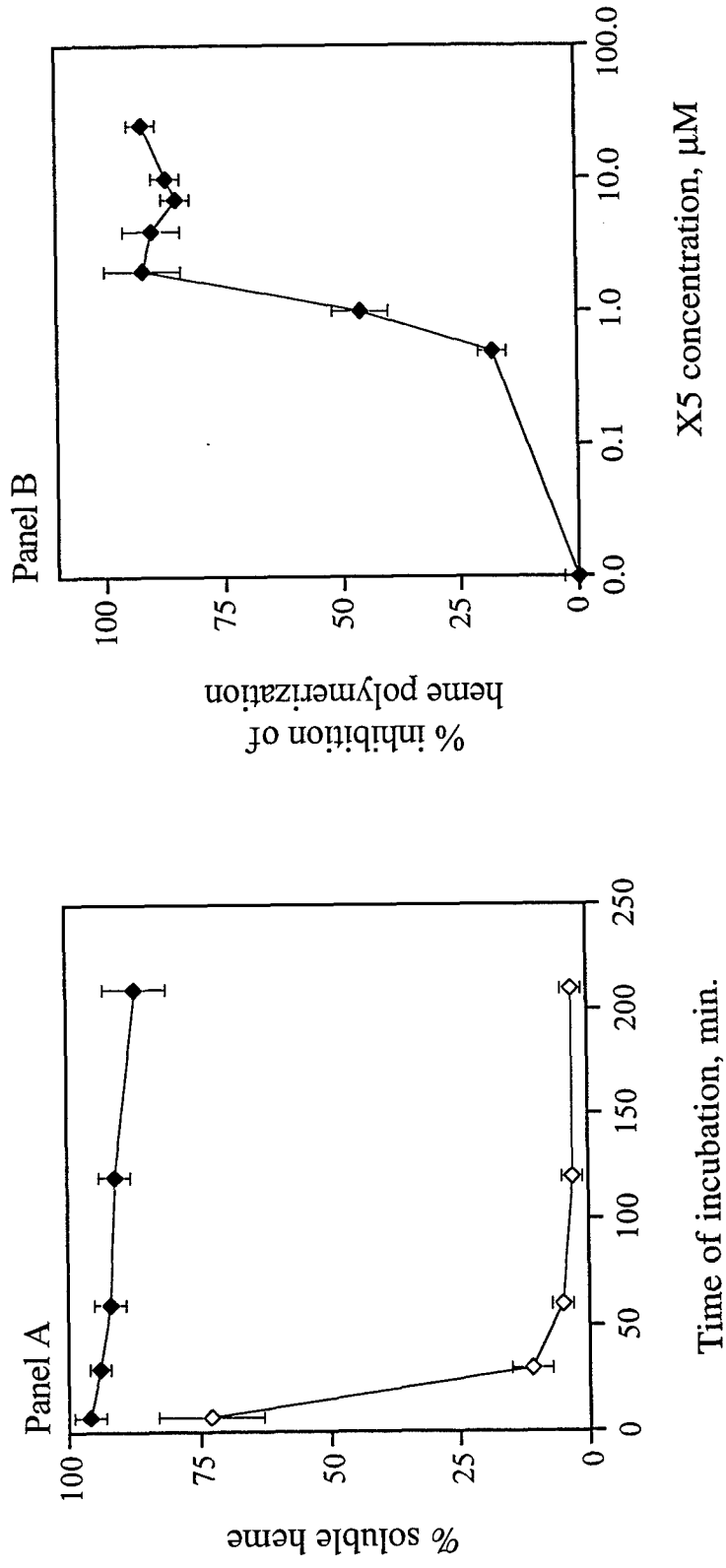
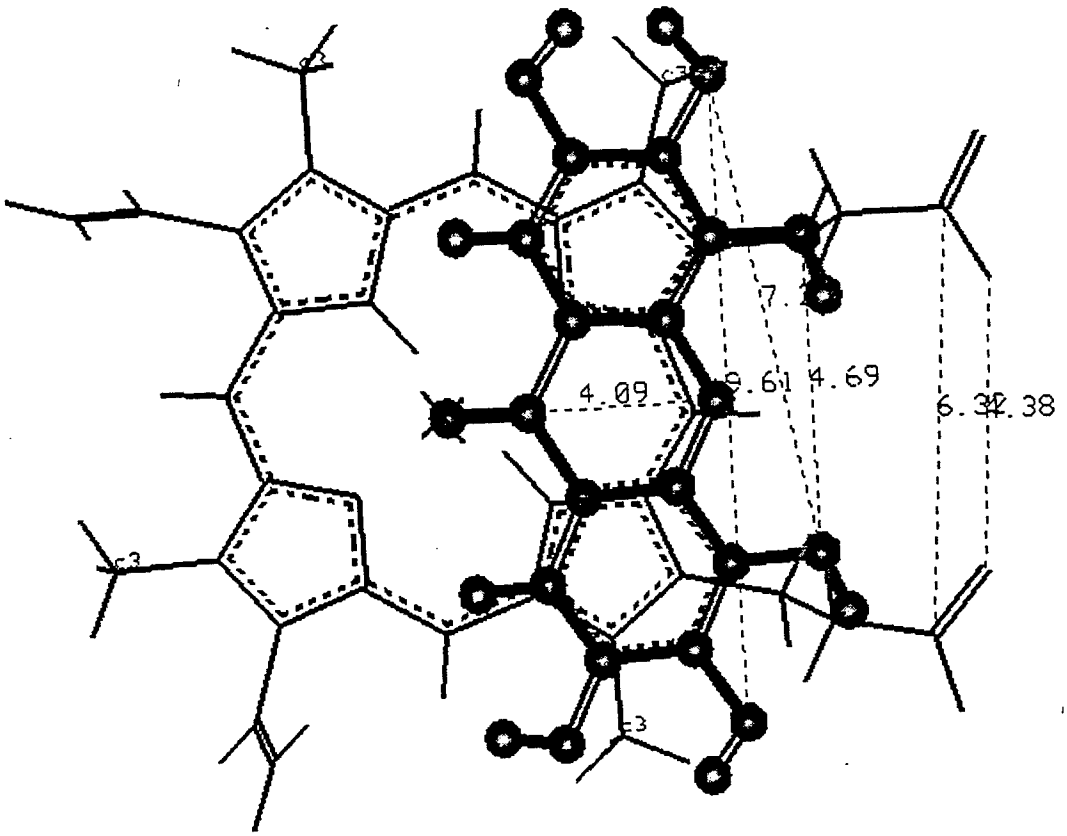


Figure 12, Panel A. Spontaneous heme polymerization in 0.02 M phosphate (pH 5.2) at 37oC in the absence (open diamonds) and presence (black diamonds) of X5. . Values are the mean + standard deviation of three independent experiments. Panel B. Dose-response effect of X5 on spontaneous heme polymerization in 0.02 M phosphate (pH 5.2) at 37oC. Initial heme concentration is 25 µM.

Figure 13. Computer simulation of X6, 2,3,4,5,6,7-hexahydroxyxanthone, docking to free heme.



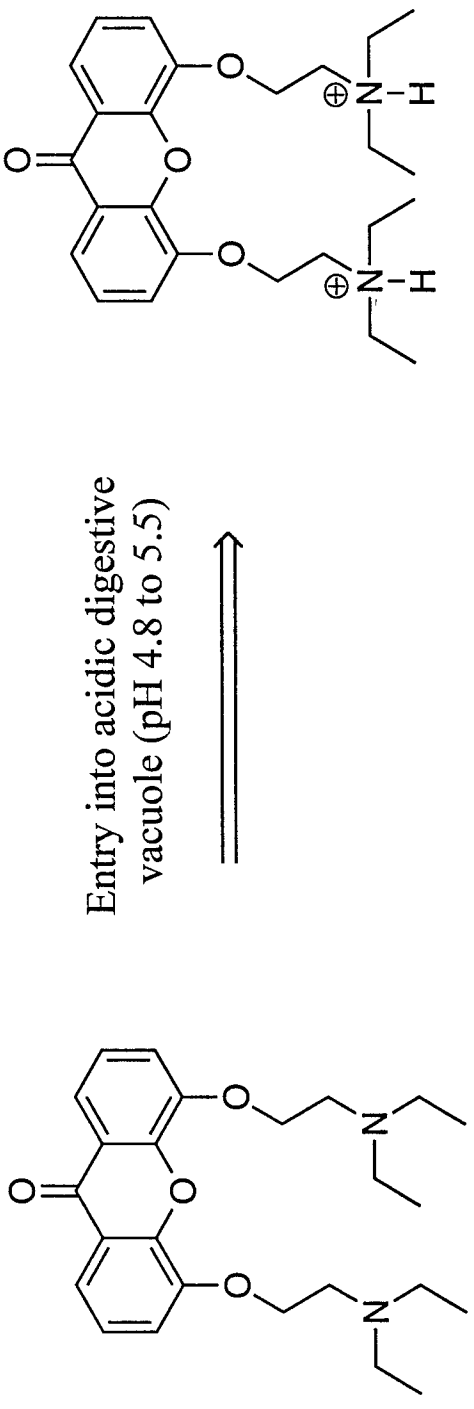


Figure 14. Structure of bis-4,5-ß-(diethylaminoethoxy)xanthone (45-DEAE-X) and formation of diprotonated form on entry into the parasite digestive vacuole.

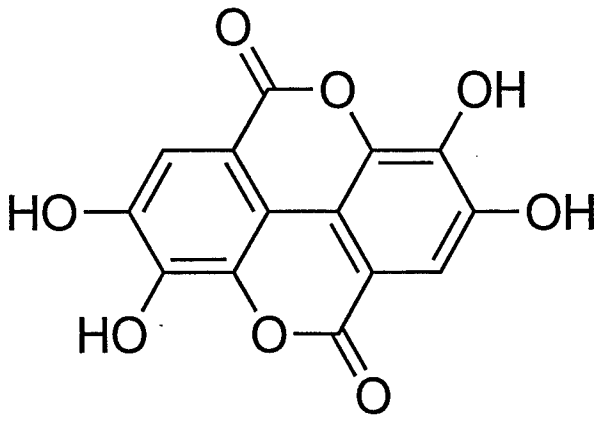


Figure 15. The chemical structure of ellagic acid.

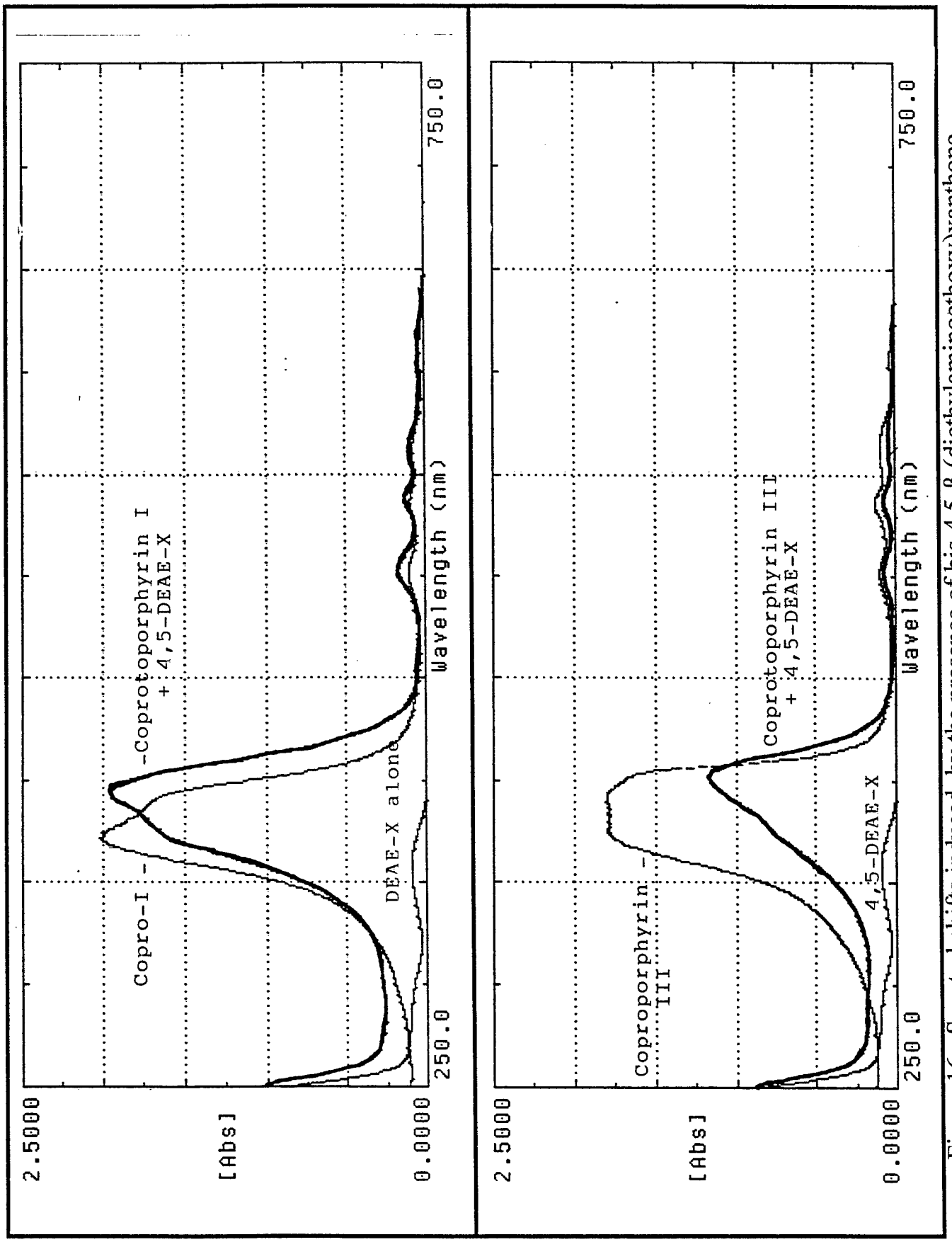
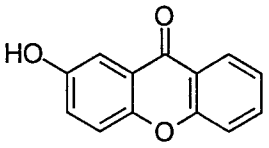
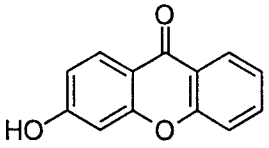
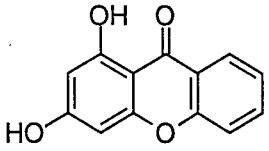
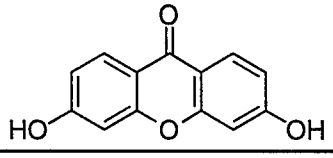
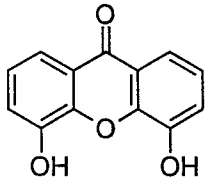
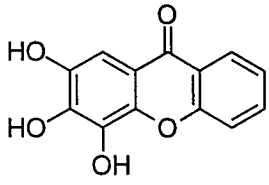
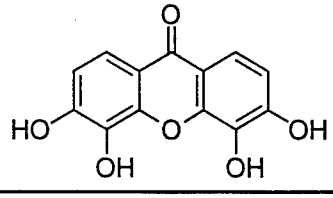
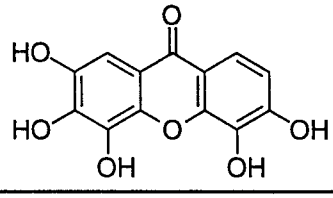
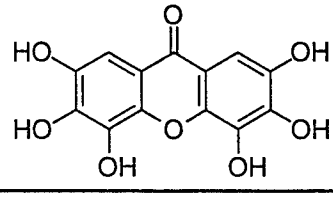
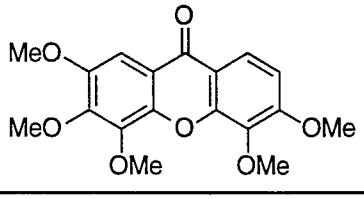


Figure 16. Spectral shifts induced by the presence of bis-4,5-ß-(diethylaminoethoxy)xanthone (4,5-DEAE-X) for coprotoporphyrin I (top) and III (bottom).

Table 1. Inhibition of *in vitro* heme polymerization by xanthones.

Compound name	Compound structure	IC_{50} µM, *P. falciparum* clone D6	IC_{50}, µM *in vitro* heme polymerization
2-hydroxyxanthone		50	>1000
3-hydroxyxanthone		>100	>1000
1,3-dihydroxyxanthone		>100	>1000
3,6-dihydroxyxanthone		>100 (INTLB) >50 (WRAIR) for both D6 and W2	>500
4,5-dihydroxyxanthone		16	14
2,3,4-trihydroxyxanthone		40	17
3,4,5,6-tetrahydroxyxanthone		5µM prep. #1 0.7µM prep. #2 3.6 (D6) & 2.6 (W2) prep. #1 (WRAIR)	2.5 (prep. 2)
2,3,4,5,6-pentahydroxyxanthone (X5)		0.4	1.2
2,3,4,5,6,7-hexahydroxyxanthone (X6)		0.1	1.4
2,3,4,5,6-pentamethoxyxanthone		>100	>1000

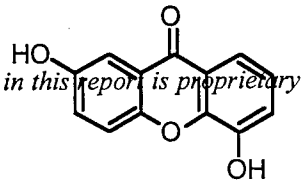
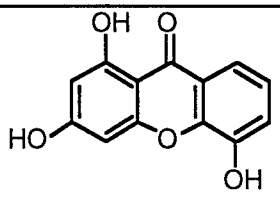
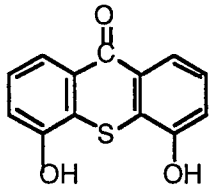
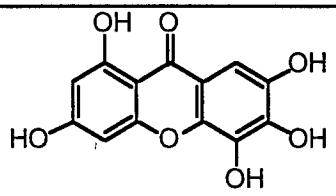
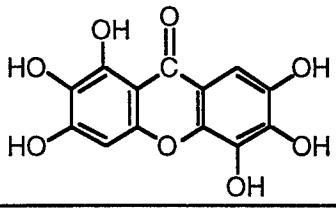
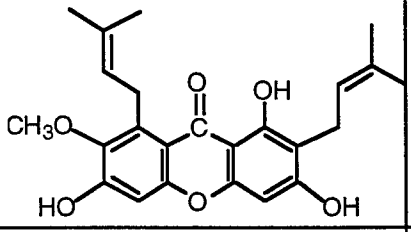
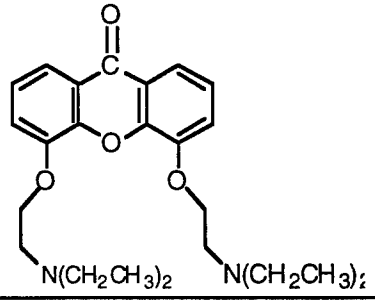
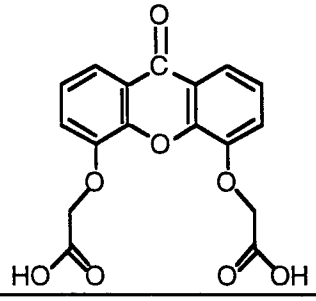
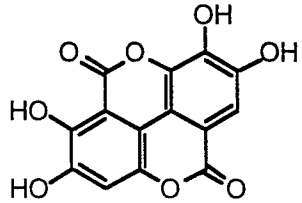
2,5-dihydroxyxanthone		>100μM	50μM
1,3,5-trihydroxyxanthone		>60	>100μM
4,5-dihydroxythioxanthone		38μM	~100μM
1,3,5,6,7-pentahydroxyxanthone ("isoX5")		7 (INTLB) 8.6 (WRAIR) & 2.4 vs. W2 (WRAIR)	9μM
1,2,3,5,6,7-hexahydroxyxanthone ("isoX6")		54	9μM
mangostin		2.5 (INTLB) 0.5 (WRAIR) & 0.7 vs. W2 (WRAIR)	23μM
4,5-bis-(ß-diethylamino-ethoxyxanthone (DEAE-X)		1.2μM* a single determination with newest material, data obtained on 6/4/97	no inhibition at equimolar concentration with heme-25μM, prelimanary indications are that some of the drug co-precipitates with a greenish product
4,5-bis-(ethyl oxyacetate)xanthone		Will be tested in the near future along with corresponding amide and amidino derivative	Not tested
ellagic acid		~0.1μM	7μM

Table 2. Physical and chemical properties of heme and heme polymers.

Sample	Solubility					Elemental composition				
	Methanol	Ethanol	Methanol/ acetic acid/ water (8:1.5:0.5)	SDS (2.5%)	Dimethyl sulfoxide	%C	%H	%N	%Fe	%H
Hematin	+	+	+	+	+	64.5	5.3	8.8	8.8	0
Malarial hemozoin[a,b]	-	-	-	-	-	64.6±0.8[c]	5.2±0.2[c]	8.7±0.2[c]	8.7±0.2[c]	ND
Hematin-phosphate (0.02 M) incubation product	-	-	-	-	-	64.3±0.3	5.4±0.3	8.4±0.3	8.8±0.5	<0.
Hematin-acetate (0.02 M) incubation product	±	±	±	-	-	64.4±0.3	5.3±0.3	8.4±0.3	8.4±0.5	<0.
Hematin-acetate (4 M) incubation product	+	+	+	+	+	59.3±0.3	5.2±0.3	7.2±0.3	7.0±0.5	<0.

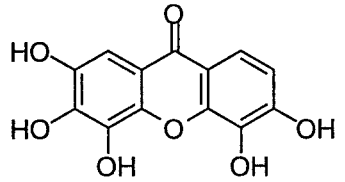
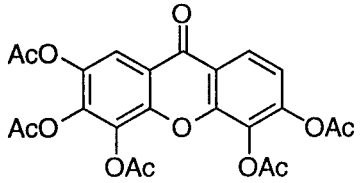
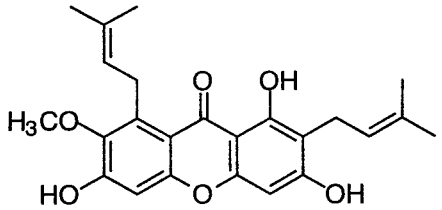
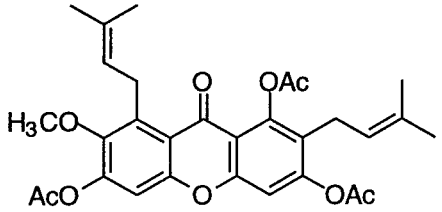
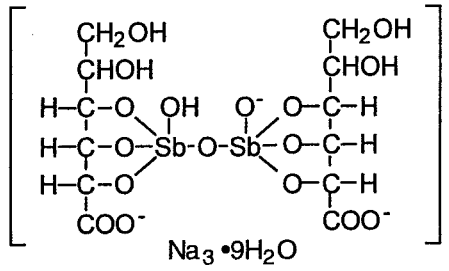
a - Pandey et al., 1996
b - Slater et al., 1991;
c - Fitch et al., 1987
d - ND, not determined

Table 3. In vitro ability of antimalarial compounds to inhibit heme polymerization and to co-precipitate with the polymerizing heme.

Compound name	Inhibition of *in vitro* heme polymerization	Co-precipitation with heme polymer *in vitro*
Artemisinin	-	ND
Chloroquine	-	+ (24%)
Methylene blue	-	+ (37%)
Primaquine	-	+ (21%)
Quinine	-	nd
Quinidine	-	nd
Quinacrine	-	(50%)
Desipramine	-	nd
Verapamil	-	nd
Xanthone	-	nd
Thioxanthone	-	nd
9-(10H)-acridone	-	nd
Quercetin	-	+
4-hydroxy-coumarin	-	nd
2',3',4'-trihydroxy-chalcone	-	+
7,8,3',4'-tetrahydroxyflavone	-	+
Exifone	+	-
Rufigallol	-	nd
Oxytetracycline	-	-
Tetracycline	-	nd
Minocycline	-	nd
Doxycycline	-	nd

Note: Co-precipitation phenomenon determined spectroscopically.

Table 4. Comparative antiparasitic effects of xanthones in vitro.

Compound	Xanthone Structure	IC_{50}, µg/ml *L. donovani* promastigotes	IC_{50}, µg/ml *L. mexicana* promastigotes	IC_{50}, µg/ml *L. tropica* 1063 promastigotes
X5		1.5	1.5	1.5
pentaacetylX5		1.5	1.5	1.5
mangostin		0.41	0.41	0.41
mangostin triacetate		nt	nt	nt
stibogluconate		~400	~400	~400

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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